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SOIL & FERTILIZER

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On the Metabolism of D-Glucuronic Acid by *Pseudomonas ovalis*

Part I. Preliminary Research with Inhibitors*

By Toshinobu ASAI and Seiichi NASUNO

Institute of Applied Microbiology, University of Tokyo

Received August 30, 1957

The enzyme systems concerning with the degradation of D-glucuronic acid by *Pseudomonas ovalis* 40F were investigated by the experiments using inhibitors for various enzyme systems. It was ascertained that the oxidation proceeded adaptively to the substrate and dehydrogenase systems containing DPN or Flavine as a coenzyme, oxidative decarboxylation and oxidative phosphorylation were presumed to participate in the oxidation of D-glucuronic acid by the phenomena of inhibition with fluoride, monoiodoacetate, arsenite, 2,4-dinitrophenol and azide to glucuronic acid oxidation. Moreover, the existence of succinodehydrogenase was also suggested by inhibition with malonate to succinic acid oxidation.

INTRODUCTION

Although it has been well known that D-glucuronic acid plays an important role in the antidotal action in the animal body and its antidotal mechanism has hitherto been studied, the metabolism of this sugar acid, itself, has yet been scarcely investigated. Above all, only a few reports have appeared in the past on oxidative metabolism of this acid by microorganisms.

Cohen[1] reported that cells of *Escherichia coli* strain K12 adapted to glucuronic acid were capable of fermentation of uronic acids, but not homologous pentoses. According to Heald[2] the fermentation pattern of D-glucuronic acid by coli form bacteria was quite different from either that of homologous hexoses or pentoses. Furthermore, Heald[3] showed that cells grown on glucose formed adaptive enzyme systems for uronic acids, but these cells were not adapted to xylose in mannometric experiments. In this latter paper it was noted that washed cell suspensions of *Aerobacter aerogenes* grown on xylose did not ferment D-glucuronic acid. Whereas on the other hand, De Ley[4] found that *Aerobacter cloacae* oxidized D-glucuronic acid after short lag periods, indicating the formation of adaptive enzyme. In studies with cells or cell-free extracts of *Escherichia coli* strain K12 grown on D-glucuronic acid, Watanabe and Arai[5] demonstrated that D-glucuronic acid was first converted to D-galacturonic acid and then to 5-keto-L-galactonic acid. In contradiction to this work, Payne[6] found that prolonged culture of *Serratia marcescens* on either D-glucuronic or D-galacturonic acid yielded cells capable of oxidizing D-glucuronic acid rapidly and D-galacturonic acid slowly. From the behavior of the D-galacturonic acid grown cells, he suggested that D-galacturonic acid was isomerized to D-glucuronic acid before being metabolized by *S. marcescens.*

Sasty and Sarma[7] showed that *Aspergillus niger* was capable of utilizing D-glucuronic acid for the precursor of L-ascorbic acid.

The present work was undertaken with

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* The present work was aided by a grant in aid defrayed from the Scientific Research Fund of the Ministry of Education.

[1] S.S. Cohen, *J. Biol. Chem.*, **177**, 607 (1949).

[2] P.J. Heald, *Biochem. J.* (London), **50**, 503 (1952).

[3] P.J. Heald, *Biochem. J.* (Lnndon), **52**, 378 (1952).

[4] J. De Ley, *Enzymologia*, **16**, 14 (1953).

[5] M. Watanabe and M. Arai, Lecture delivered at the annual meeting of Japanese Biochemical Society, Nov. 2 (1955).

[6] W.J. Payne, *J. Bact.*, **72**, 834 (1956).

[7] K.S. Sasty and P.S. Sarma, *Nature*, **179**, 44 (1957),

the view to obtain information refering to the mechanism of D-glucuronic acid oxidation by *Pseudomonas ovalis* as a part of series of studies on oxidative fermentation of bacteria.

EXPERIMENTAL

A strain of *Pseudomonas ovalis* 40F** isolated by Yokozawa8) was selected as the test organism from about forty strains of *Acetobacter*, *Gluconobacter* and *Pseudomonas*. This strain is a facultative aerobe which oxidizes glucose by way of gluconic acid. It produces neither 2-keto-gluconic nor 5-keto-gluconic acid and has no ability to oxidize either of them. It is also remarkably different from another strain of *Pseudomonas* such as *Ps. aeruginosa* and *Ps. fluorescens liquefaciens* in this respect9)10). Cells of *Acetobacter* and *Gluconobacter* tested were not induced to oxidize D-glucuronic acid, nor did extention of the period of incubation to 5 hours result in adaptation by them. Cultures were grown in shake culture on a reciprocal shaker at pH 5.8 in a medium containing 0.3% dried yeast extract powder (Oriental Yeast Manuf. Co.), 10% glucose and 2.5% calcium carbonate. The cells were harvested at a period of 18 hours, washed with 1.5% sodium chloride solution and distilled water. To prepare cells adapted to D-glucuronic acid, they were finally suspended at a concentration of 100 mg wet weight per ml in M/20 phosphate buffer (pH 6.0) containing 0.7% sodium D-glucuronate and were incubated at 30°C for 5 hours. Cells adapted were rewashed as described above and taken up in distilled water to give a suspension of about 25 mg dry weight per ml (except those employed in the experiments shown in Fig. 1). Oxygen uptake and CO_2 evolution were measured by the Warburg apparatus at 30°C. All the values of endogenous respiration were subtracted. Each mannometer cup was charged with one ml of M/20 phosphate buffer (pH 6.0, except in case to test the effect of pH), 0.5 ml of substrate (5 μM), 0.5 ml of bacterial suspension in the side arm and 0.2 ml of 20% potassium hydroxide in the center well. Moreover, a portion of 0.5 ml of aqueous solution of the inhibitor (whose concentration was in the figures respectively) or an equal volume of water was added to the cup in the experiments in order to examine the effects of inhibitors. Inhibitors used in this study were sodium fluoride, monoiodoacetic acid, sodium arsenite, 2,4-dinitrophenol, sodium azide and malonic acid. Monoiodoacetic and malonic acids were neutralized with sodium hydroxide before being employed. They were all extra-pure products obtained commercially.

RESULTS AND DISCUSSION

Washed cells of *Ps. ovalis* 40F grown on glucose exhibited adaptation to D-glucuronic acid after incubation for 2 hours (Fig. 1, curve A) like *E. coli*1)5), coli form bacteria3), *Aero. cloacae*4) and *S. marcescens*6) investigated hitherto did. Reaction mixtures used in the experiment by which curve A in Fig. 1 was obtained were centrifuged to collect the cells. The cells were used for measuring the oxygen uptake and resulted curve B in Fig. 1. In this latter experiment D-glucuronic acid was oxidized without a lag period and linearly to incubation period. The rate of oxidation of D-glucuronic acid by adapted cells increased up to several times to that of the cells

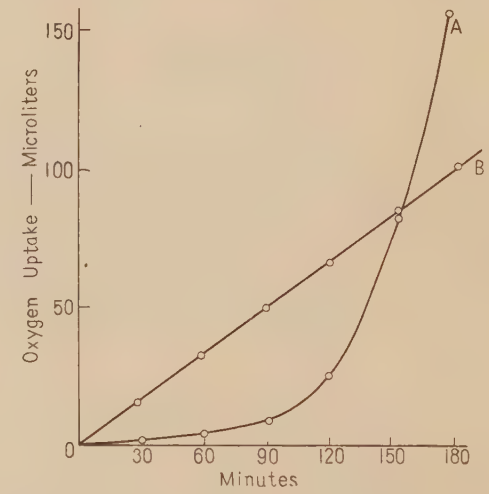

FIG. 1. Adaptive Curves of D-Glucuronic Acid Oxidation.

** This strain was formerly designated as *Ps. fluorescens* 40F by Yokozawa, however, it has been corrected as *Ps. ovalis* according to the exact identification by Shoda in our laboratory.

8) K. Yokozawa, *J. Agr. Chem. Soc. Japan*, **25**, 556 (1951).

9) J.J.R. Campbell and F.C. Norris, *Canad. J. Research*, C28, 203 (1950), F.N. Stokes and J.J.R. Cambell, *Arch. Biochem. Biophys.*, **30**, 121 (1950).

10) T. Asai and H. Murooka, *J. Agr. Chem. Soc. Japan*, **30**, 1 (1956).

before adaptation occurred.

The effect of pH to the oxidation of D-glucuronic acid is shown in Fig. 2. Although optimal pH was found to be approximately 7, little difference on enzyme activities was observed between pH 7 and pH 6 or 8. Thus, pH 6 was conventionally selected in the experiments hereafter.

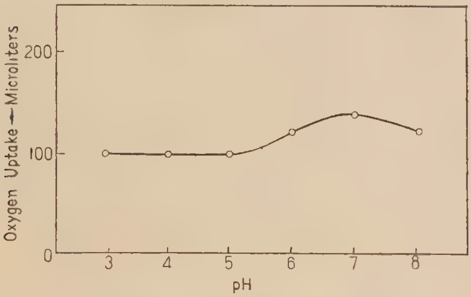

FIG. 2. Optimum pH in the Oxidation of D-Glucuronic Acid.

The Q_{O_2} value for D-glucuronic acid was about 25–30 and RQ value was 1 at these pH conditions.

The metabolism of D-glucuronic acid in microorganisms has scarcely been investigated, and especially, its terminal pathway has been completly unclarified. Therefore, the inhibition phenomena to various enzyme systems with inhibitors were investigated to presuppose it.

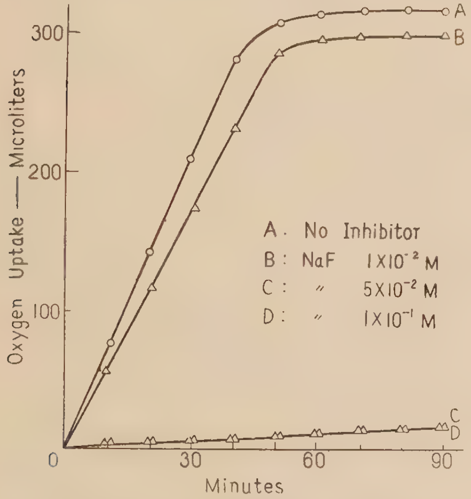

FIG. 3. Inhibition to D-Glucuronic Acid Oxidation with NaF.

Sodium fluoride at a concentration of 5×10^{-2} M completely inhibited the oxidation of D-glucuronic acid (Fig. 3). Oxygen uptake to D-glucuronic acid by intact cells was perfectly suppressed with sodium monoiodoacetate at a minimum concentration of 5×10^{-3} M(Fig. 4). Therefore, the evidence was supported that dehydrogenase systems contained DPN or Flavin as coenzyme might participate in the oxidation of D-glucuronic acid.

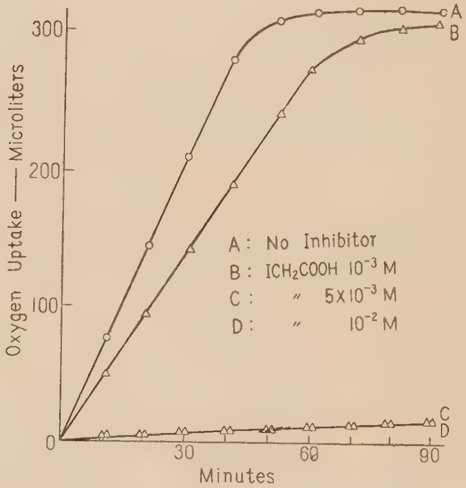

FIG. 4. Inhibition to D-Glucuronic Acid Oxidation with ICH_2COOH.

Sodium arsenite showed a remarkable inhibition at a concentration of 10^{-3} M(Fig. 5). Since arsenite inhibits oxidative decarboxylation, the existence of oxidative decarboxylation was readily assumed from this experiment.

At 10^{-3} M 2,4-dinitrophenol and at 10^{-2} M sodium azide concentrations, perfect inhibitions to D-glucuronic acid degradation were observed (Figs. 6 & 7). Both inhibitors are reagents for suppression to the oxidative

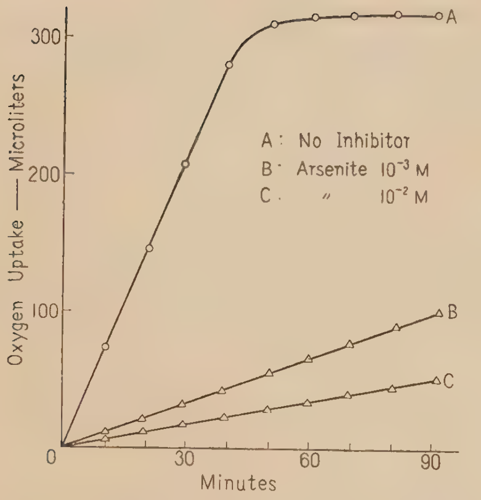

Fig. 5. Inhibition to D-Glucuronic Acid Oxidation with Arsenite.

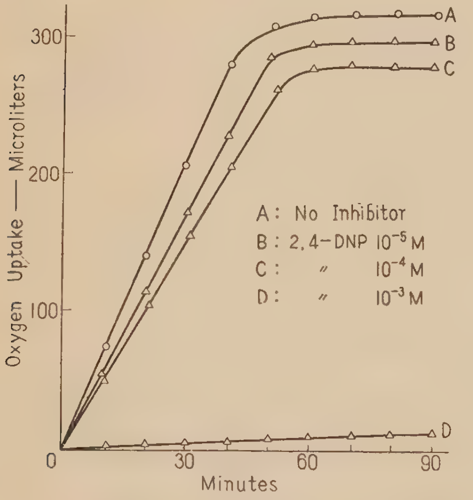

Fig. 6. Inhibition to D-Glucuronic Acid Oxidation with 2,4-DNP.

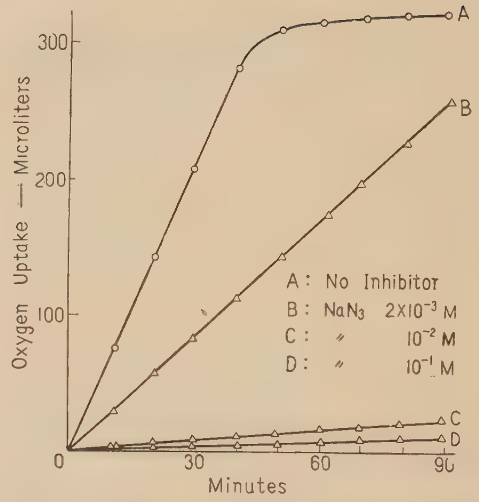

FIG. 7. Inhibition to D-Glucuronic Acid Oxidation with NaN_3.

phosphorylation in the enzyme reaction sequence. Watanabe and Arai[11] presumed that the phosphorylation in D-glucuronic acid metabolism by *E. coli* strain K12 would be occurred at the 6-position of carbon of 5-keto-L-galactonic acid. Although it was not clarified where the phosphorylation did occur, it would be said that the phosphorylation took place somewhere in the metabolic pathway of D-glucuronic acid oxidation by *Ps. ovalis*.

Sodium malonate at a minimum concentration of 10^{-2} M suppressed the oxidation of succinic acid by the resting cells of *Ps. ovalis* (Fig. 8). The incomplete inhibition might be caused by the permeability of malonate

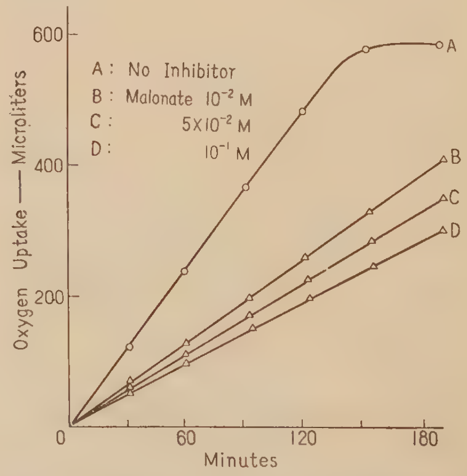

FIG. 8. Inhibition to Succinate Oxidation with Malonate.

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[11] M. Watanabe and M. Arai, Lecture delivered at the Symposium of Enzyme Chemistry, July 24 (1956).

to succinodehydrogenase in washed cells.

Since D-glucuronic acid did not produce any oxidation product by *Ps. ovalis* 40F, it might be completely oxidized. It might be caused by oxidative assimilation that the values of oxygen uptake by intact cells did not coincide to the values calculated theoretically according to the formula of complete oxidation. From this and the results obtained above, it was supposed that D-glucuronic acid was finally metabolized to CO_2 and H_2O through Krebs' TCA cycle in a manner like that observed in the glucose oxidation by another *Pseudomonas* such for instance as *Ps. aeruginosa*, *Ps. fluorescens.* Further details concerning with this problem would be investigated in the following later paper.

Acknowledgement. We are indebted to Mrs. K. Minoda for her corporation in carrying out this study.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 1, 6~10, 1958]

On the Metabolism of D-Glucuronic Acid by *Pseudomonas ovalis*

Part II. Metabolic Pathway*

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Received August 30, 1957

From the results obtained by the method of successive adaptation and paper chromatography of reaction mixtures by intact cells, it was presumed that D-glucuronic acid was first converted through D-galacturonic acid to 5-keto-L-galactonic acid and then further oxidized. The accumulation of pyruvic acid was observed in the reaction mixtures under aerobic incubation when the enzyme system was blocked with sodium arsenite. Moreover, α-ketoglutaric acid was also found in the reaction mixtures with D-glucuronic or D-galacturonic acid as a substrate under aerobic incubation. Resting cells of the tested organisms showed the ability to oxidize all members of Krebs' tricarboxylic acid cycle, pyruvic and acetic acids. It is therefore proposed that the oxidation of D-glucuronic acid by *Pseudomonas ovalis* assumes the following pathway:

D-glucuronic acid ⟶ D-galacturonic acid ⟶ 5-keto-L-glactonic acid ⟶ unknown ⟶
pyruvic acid ⟶ Krebs' tricarboxylic cycle ⟶ CO_2+H_2O

INTRODUCTION

Many informations on the metabolism of D-glucuronic acid by animal tissue enzymes have long appeared during the past years. Several pathways involving the metabolic conversion of glucuronolactone to L-xylulose and D-ribose have been reported by Enklewitz1), Touster2) and Horecker3). Recently, Rabinowitz and Sall4) obtained the result that the particle free enzyme system of rat kidney yielded approximately 12% of CO_2 which was predominantly derived from the sixth position of carbon of glucuronolactone and D-glucuronic acid. However, it has scarcely been known on the metabolic pathway of D-glucuronic acid by microorganisms.

Cohen5) reported that *E. coli* K12 strain adapted to D-glucuronic acid did not produce D-xylose. The same result was obtained by Heald6) with coli-form bacteria. Heald7) also showed that formic, acetic and lactic acids were found as the products of fermentation from D-glucuronic acid.

Payne8) suggested that dried cells of *Serratia marcescens* grown on glucose minimal medium and adapted to D-glucuronic acid yielded a 1,6-"ester" linked dihexuronic acid from buffered D-glucuronic acid. This substance might be supposed to be an intermediate in the oxidative metabolism of D-glucuronic acid by this bacteria. He also discussed that D-galacturonic acid was isomerized to D-glucuronic acid before being metabolized *S. marcescens*.

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* The present work was aided by a grant in aid defrayed from the Scientific Research Fund of the Ministry of Education.

1) M. Enklewitz and M. Lasker, *J. Biol. Chem.*, **110**, 443 (1953).

2) O. Touster, R. H. Mutcheson and L. Rice, *J. Biol. Chem.*, **215**, 677 (1955).

3) B. L. Horecker, P. Z. Smyrnoitis and J. E. Seegmiller, *J. Biol. Chem.*, **193**, 383 (1951).

4) J. L. Rabinowitz and T. Sall, *Biochem. Biophys. Acta*, **23**, 289 (1957).

5) S. S. Cohen, *J. Biol. Chem.*, **177**, 607 (1949).

6) P.J. Heald, *Biochem. J.* (London), **50**, 503 (1952).

7) P.J. Heald, *Biochem. J.* (London), **52**, 378 (1952)

8) W J. Payne, *J. Bact.*, **72**, 834 (1956).

In contradiction to this hypothesis, Watanabe and Arai[9] ascertained that D-glucuronic acid was isomerized to D-galacturonic acid and then was converted to 5-keto-L-galactonic acid before being metabolized by strain K12 of *E. coli*.

In this paper we present a presumable pathway of the oxidation of D-glucuronic acid by *Ps. ovalis* 40F in which D-glucuronic acid was converted to 5-keto-L-galactonic acid via D-galacturonic acid and was oxidized through pyruvic acid and Kreb's tricarboxylic acid cycle to CO_2 and H_2O.

EXPERIMENTAL

The cell preparation adapted to D-glucuronic acid and the mannometric procedures were the same as previously described[10].

The paper chromatographical techniques were employed to detect the intermediary products in the oxidation of D-galacturonic acids. The papers were developed by the descending method with upper layer of a solvent mixture consisting of 4 volumes of *n*-butanol, 1 volume of acetic acid and 5 volumes of water. As a reagent for the detection of reducing sugars and reducing sugar acids, 3% *n*-butanol solution of *p*-anisidine hydrochloride was used. To detect organic acids derived from D-glucuronic acid, 1% alcohol solution of Brom Phenol Blue was applied. Keto-acids such as α-ketoglutaric and pyruvic acids were derived to the 2, 4-dinitrophenylhydrazones of them in order to observe yellow spots on the developed paper. They were always compared with authentic samples developed simultaneously.

Aerovic and anaerobic incubations containing a large amount of substrate with intact cell suspension were run to catch intermediary products in the oxidation of D-glucuronic and D-galacturonic acids and were tested quantitatively by the paper chromatographical methods at the interval of half an hour.

The reaction mixtures were prepared to contain 1.5 mM substrate as sodium salt, 30 ml of M/20 phosphate buffer at pH 6.0 and 30 ml of bacterial suspension containing 360 mg dried cells. Under anaerobic incubation, the atmosphere in the vessel was exchanged with N_2 gas.

Spot tests[11] were applied to check formic and acetic acids in the reaction mixtures after incubation.

In order to accumulate pyruvic acid by blocking enzyme system, sodium arsenite in a concentration of 10^{-3} M was used with the substrate of D-glucuronic or D-galacturonic acid.

A successive adaptation method was used to support the fact that D-galacturonic and 5-keto-L-galacturonic acids were the intermediary products in the metabolic sequences of D-glucuronic acid degradation. Furthermore, the ability to oxidize several substances which were presumed as intermediates, all members of Krebs' tricarboxylic acid cycle, pyruvic and acetic acids were tested by the respirometery.

5-Keto-L-galactonic acid was prepared by the method of Ehrlich and Gutterman[12]. 2-Keto-D-galactonic acid was kindly supplied by Assist. Prof. K. Aida of our laboratory. The other chemicals used were commercial products

RESUTL AND DISCUSSION

The reaction mixture, which was incubated for 2 hours anaerobically with D-glucuronic acid as a substrate, was centrifuged after it was deprotenized by adding 50% trichloroacetic acid. Its supernatant was spotted on the paper and developed by the descending method. Four spots were found after spraying with 3% *n*-butanol solution of *p*-anisidine hydrochloride and their R_F values were 0.34, 0.17, 0.18 and 0.23 respectively. The former two were brown, the third was pink and the fourth was yellow. They corresponded to glucuronolactone, D-glucuronic, D-galacturonic and 5-keto-L-galactonic acids, although the existence of glucuronolactone was doubtful by spraying with 1% alcohol solution of Brom Phenol Blue. Furthermore, the spot of 5-keto-L-galactonic acid was also observed in the reaction mixture containing D-galacturonate as a substrate under same condition.

The method of successive adaptation by intact cells was applied to ratify that these two substances are intermediary metabolites by testing organism adapted to D-glucuronic acid. The result is shown in Figure 1.

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9) M. Watanabe and M. Arai, Lecture delivered at the annual Meeting of Japanese Biochemical Society, Nov. 2 (1955).

10) T. Asai and S, Nasuno, This Bulletin, **22**, 1 (1958)

11) F. Feigl, Spot Tests II, p. 245, 247 (1954).

12) F. Ehrlich and R. Gutterman, *Ber.*, **67**, 573 (1934).

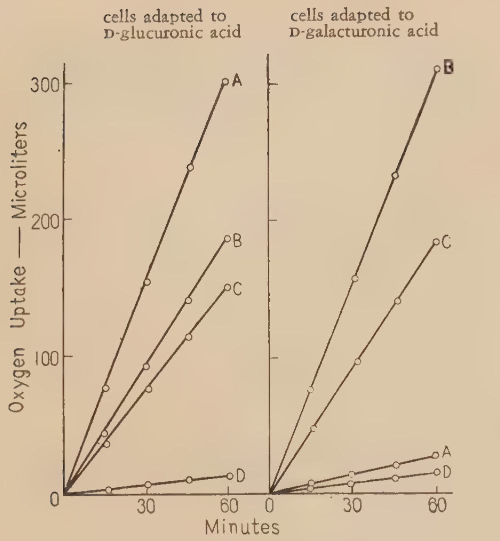

FIG. 1. Successive Adaptation to D-Glucuronic (A), D-Galacturonic (B), 5-Keto-L-Galactonic (C) and 2-Keto-D-Galactonic Acid (D).

Although both D-galacturonic and 5-keto-L-galactonic acids were oxidized after short lag periods by the cells not adapted to D-glucuronic acid, they were immediately oxidized without lag periods by the cells adapted to D-glucuronic acid. And furthermore, D-glucuronic acid was not oxidized, but D-galacturonic and 5-keto-L-galactonic acids were rapidly oxidized by the cells adapted to D-galacturonic acid. Nor was 2-keto-D-galactonic acid completely oxidized by the cells adapted to either D-glucuronic or D-galacturonic acid.

The primary-stage of conversion of D-glucuronic acid by *Ps. ovalis* appeared to be very similar with the result obtained by *E. coli* which was reported by Watanabe and Arai13).

Several sugars and sugar acids being anticipated as intermediary products in the oxidation of D-glucuronic acid were checked by the mannometric method (Fig. 2). In addition to this, these tested sugars were not detected in the reaction mixture containing either D-glucuronic or D-galacturonic acid as a substrate, so from these experiments it seemed that D-arabinose, D-xylose and D-ribose have no such possibility. L-Xylulose and D-ribose have been reported as metabolites by Enklewitz1), Touster2) and Horecker3). Heald7) showed that cells grown on D-glucuronic acid could not ferment galactose, xylose and arabinose by coli-form bacteria. Hough and Pridham14) reported that no evidence was obtained for the conversion of the lactone of D-glucuronic acid to D-xylose by decarboxylation process with plum mesocarp tissues.

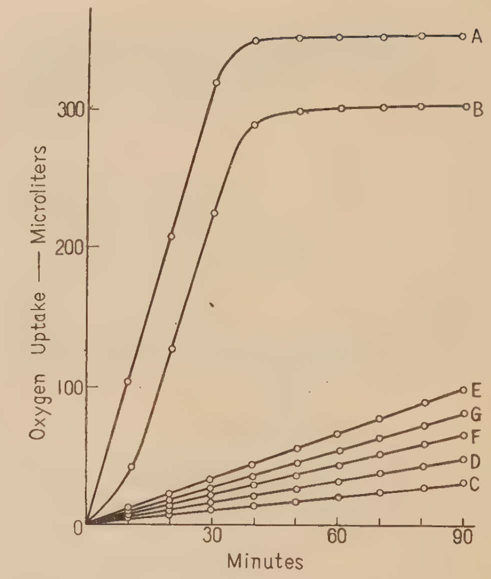

FIG. 2. The Oxidation of Glucose (A), Gluconic (B), 2-Ketogluconic (C) Acids, Galactose (D), Arabinose (E), Xylose (F) and Ribose (G).

In order to catch the intermediary products in the rather terminal stage of metabolic pathway of D-glucuronic acid, reaction mixtures containing materials the same as those used in anaerobic incubation were incubated under aerobic condition. Samples of 10 ml were taken at 15 minutes intervals and mixed with equal volumes of acetone to stop the

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13) M. Watanabe and M. Arai, Lecture delivered at the Symposium of Enzyme Chemitry, July 24 (1956).

14) L. Hough and J.B. Pridham, *Arch. Biochem. and Biophys*, **59**, 17 (1955).

reaction. The samples were centrifuged and a part of supernatant from each vessel was tested to detect acetic and formic acids by the technique of spot tests, but these volatile acids could not be found contrary to Heald's report7). Another part of the supernatant was taken to prepare 2, 4-dinitrophenylhydrazones of keto acids to apply chromatography. The *n*-butanol, acetic acid solvent was permitted to irrigate the chromatograms. Since, 2, 4-dinitrophenylhydrazone of α ketoglutaric acid was detected in a sample at 30 minutes, α-ketoglutaric acid was supposed to be a possible intermediate of D-glucuronic acid metabolism.

The reaction mixtures containing either D-glucuronic or D-galacturonic acid as a substrate and sodium arsenite as a inhibitor were treated by the procedure as same as that of aerobic incubation mixtures. 2, 4-Dinitrophenylhydrazone of pyruvic acid was detected on the paper chromatogram, so pyruvic acid was also ascertained as a metabolic intermediate of the degradation of D-glucuronic acid.

It has been investigated rather well that genus *Pseudomonas* have the enzymes of the Krebs' tricarboxylic acid cycle. According to Campbell and Stokes15), and Campbell and Smith16), both resting and dried cells of *Ps. aeruginosa* possess the enzymes necessary for the oxidation of compounds of the tricarboxylic acid cycle. They revealed that the limiting factor in the immediate utilization of these intermediates by resting cells was the impermeability of the cell membranes. Robinson and Katznelson17) reported that all the enzymes of the tricarboxylic acid cycle, with the exception of oxalosuccinic acid decarboxylase, were detected in sonic extracts of *Ps. nigrificans.*

Resting cells harvested from a glucose medium and adapted to D-glucuronic acid had no ability to attack *cis*-aconitic, citric

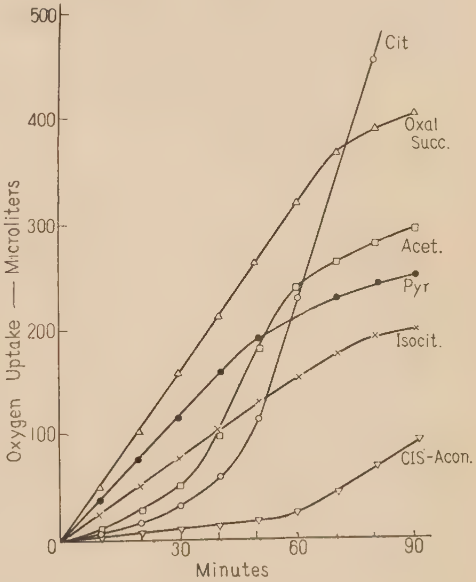

FIG. 3. The Oxidation of Citric, *cis*-Aconitic, *iso*-Citric, Oxaloacetic, Acetic and Pyruvic Acids.

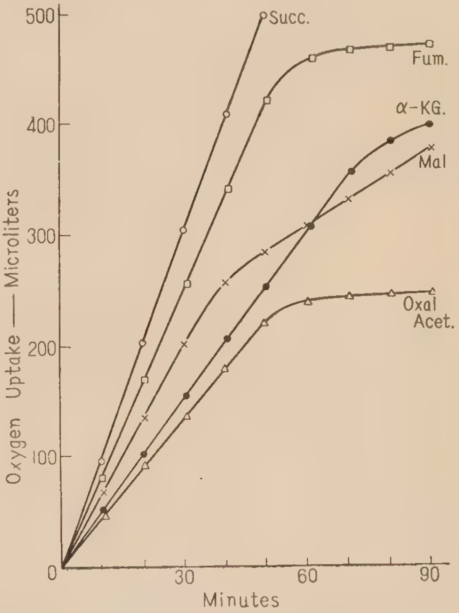

FIG. 4. The Oxidation of α-Ketoglutaric, Succinic, Fumaric, Malic and Oxaloacetic Acids.

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15) J. J. R. Campbell and F. N. Stokes, *J. Biol. Chem.*, **190**, 853 (1951).

16) J. J. R. Campbell and R. A. Smith, *Canad. J. Microbiol.*, **2**, 433 (1956).

17) J. Robinson and H. Katznelson, *Canad. J. Microbiol.*, **2**, 723 (1956).

and acetic acids immediately (Fig. 3 and 4). However, activity was demonstrated with other members of the tricarboxylic acid cycle and pyruvic acid. The presence of the enzymes necessary for the oxidation of the members of tricarboxylic acid cycle in the cells of *Ps. ovalis* points to the constitutive nature of the enzymes involved.

It is now known that *Ps. ovalis* oxidizes D-glucuronic acid by way of D-galacturonic and 5-keto-L-galactonic acids. Since pyruvic and α-ketoglutaric acids were ascertained as intermediates in the oxidation of D-glucuronic acid, it is likely that the oxidation of these intermediates are of supreme importance as a means of oxidizing D-glucuronic acid by way of the Krebs' tricarboxylic acid cycle. As there is no evidence where esterification of phosphate does occur, it is therefore, vague to find now 5-keto-L-galactonic acid degradates to pyruvic acid. However, synthesizing the results obtained above, a presumable pathway of the oxidation of D-glucuronic acid by *Ps. ovalis* was strongly supposed as shown in Figure 5.

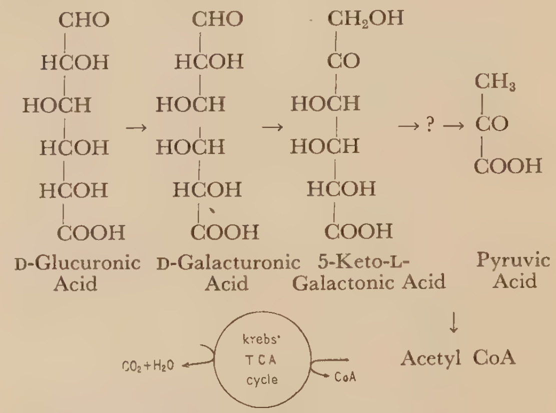

FIG. 5. Presumable Pathway of D-Glucuronic Acid Oxidation by *Pseudomonas ovalis* 40F.

Acknowledgement. We sincerely wish to express our appreciation to Mrs. K. Minoda for her corporation in carrying out this study.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 1, p. 11~13, 1958]

Studies on Mannan in Wood Pulp

Part II. 4-O-β-D-Glucosyl-α-D-Mannose Obtained from Dissolving Pulp

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Wood Research Institute, Kyoto University

Received September 2, 1957

Octaacetyl 4-O-β-D-glucosyl-α-D-mannose was isolated by application of "Magnesol" chromatography from the acetolyzate of commercial dissolving pulp. This fact indicates the existence of the chemical linkages of mannose units to glucose residues in purified cellulose materials manufactured from softwood.

It was assumed in the previous paper[1], that at least, a part of mannan is present in wood pulp as glucomannan. Isolation of octaacetyl-4-O-β-D-glucosyl-α-D-mannose (α-epicellobiose octaacetate) from the acetolysis product of dissolving pulp shows the existence of the chemical linkages of mannose to glucose units in refined wood cellulose materials. Recently, epicellobiose was reported by Anthis[2] to be isolated from Leech's products of slash pine and by Jones[3], from the hydrolyzate of loblolly pine wood and, however, in spite of the fact that Anthis used wood meal containing 10% mannan as the starting material, the yield obtained was only 0.29%. The pulp used in this experiment was sulphite pulp manufactured at the Sanyo Pulp Co. Ltd., Japan, intended for the manufacture of viscose rayon containing 91.9% α-cellulose. The acetolyzate, from which was removed crystalline cellobiose octaacetate produced on the acetolysis of the pulp, was fractionated by the use of Wolfrom's technique[4]. Under the conditions described in the experimental part, a zone of epicellobiose octaacetate was located below the strongly adsorbed part on the column, and the disaccharides mainly consisting of cellobiose octaacetate, and monosaccharides elute out of the column. The yield of α-epicellobiose octaacetate was 2.91% on the acetolyzate used, which contained 8.50% mannose residues. It is therefore obvious, that a considerable amount of the mannose residues in refined pulp does not reside as mannan but in the combined form of glucose residues. This linkage seemed to have a respectable resistance to sulphite pulping. In this experiment, two unconfirmed crystalline disaccharides were isolated from the effluent part.

EXPERIMENTAL

Acetolysis of Dissolving Pulp

An amount of 20 g of dry powder of the dissolving pulp was added portionwise to a mixture of 75 ml of acetic anhydride, 75 ml of glacial acetic acid and 8 ml of concentrated sulfuric acid, which was cooled previously at −10° by external cooling with ice-salt. The temperature of the reaction mixture was held below 10° during the addition of the pulp. After standing at 30 ±2° for 10 days, the brown solution was held at 0°, until the crystallization of cellobiose octaacetate was complete and then, the crystals were filtered; yield 7.15 g. The filtrate was immediately poured onto ice-water (6 l) and neutralized with sodium bicarbonate to pH 6.0 and then allowed to stand overnight. The white precipitate was removed by filtration, washed with water until neutral to litmus and dried over calcium chloride in vacuo. This acetolyzate contained 8.50% of mannose residues.

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[1] T. Koshijima and I. Tachi, This Bulletin **21**, 253 (1957).

[2] A. Anthis, *TAPPI* **39**, No. 6, 401 (1956).

[3] D.H. Ball, J.K.N. Jones, W.H. Nicholson, and T.J. Painter, *TAPPI* **39**, No. 6, 438 (1956).

[4] W.W. Binkley and M.L. Wolfrom, "Chromatography of Sugars and Related Substance", Sugar Research Foundation, Inc., New York (1948).

Chromatographic Fractionation of Acetolyzate

Following the general procedure of Binkley and Wolfrom (loc. cot.), the acetolyzate was chromatographed on the "Magnesol" column. A quantity of 1 g of the acetolyzate in 30 ml of chloroform was added to the top of the column (43×170 mm) composed of 5 parts of "Magnesol" and 1 part of "Celite" and developed with 1 liter of benzene-*t*-butanol (65 : 1). The extruded column was streaked with alkaline permanganate solution and showed two zones, one of which was located from the top of the column to 80 mm (Fraction 1) and the other, from 120 to 160 mm (Fraction 2). The zones were separated, eluted with acetone and the eluates evaporated to dryness. From 10 g of the acetolyzate, the following yields were obtained: Fraction 1, 3.12 g; Fraction 2, 1.30 g; Effluent Fraction, 4.31 g.

Octaacetyl 4-O-β-D Glucosyl-α-D-Mannose

The hot ethanol-solution of Fraction 2. gave 291 mg of small needle crystals: m.p. 195–198°. After two recrystallizations from absolute ethanol, the melting point arised to 201.5–203° which was unchanged upon admixture with a known octaacetyl 4-O-β-D-glucosyl-α-D-mannose; yield 160 mg, $[\alpha]_D^{20}$+36.1° (c, 0.5, chloroform); Calcd. for $C_{28}H_{38}O_{19}$: C, 49.56; H, 5.64; MW, 679. Found: C, 49.47; H, 5.65; MW, 745.

A portion of the crystals (20 mg) was deacetylated with 0.2 N-sodium methoxide and hydrolyzed with N-sulfuric acid for 3 hours. Mannose and glucose were detected on the paperchromatogram of the deionized neutral solution.

The other part (41 mg) of the crystalline acetate was deacetylated with 0.4 N-barium methoxide. After deacetylation, water was added to the methanol solution, which was titrated with 0.1 N-sulfuric acid until neutral to phenolphthalein and then filtered. The filtrate was concentrated to a syrup with the addition of absolute ethanol under reduced pressure and the resultant ethanol solution was filtered again and finally dried over anhydrous calcium chloride; yield 18 mg. From this parent sugar, 12 mg of mannose phenylhydrazone was prepared in the ordinary way: m.p. and mixed m.p., 186° (decomp.). Glucose phenylosazone (11 mg) was separated from the filtrate: m.p. and mixed m.p., 198° (decomp.).

The chloro-heptaacetyl derivative of the crystalline acetate was prepared according to the procedure of Brauns[5]. A portion of the crystalline acetate (22 mg) was added to acetic anhydride (0.5 ml) and dry hydrogen chloride gas was bubbled to the solution for thirty minutes under cooling at −20°. The vessel containing clear solution was closed and allowed to stand for one week at room temperature. The white powder remaining after evaporation of the solvent crystallized with ethyl acetate-petroleum ether but not however, with ethyl acetate-ether, or chloroform-ether. yield 11 mg, m.p. 162–164°. After filtration of the insoluble matter in ethyl acetate, recrystallization with the same solvent system gave m.p. of 171–172° which was the identical with the value indicated by Brauns (loc. cit.).

From the fact described above, it was confirmed that the crystalline acetate was octaacetyl 4-O-β-D-glucosyl-α-D-mannose.

Further Chromatography of Effluent Fraction

The effluent fraction of solids was dissolved in hot ethanol and, on cooling, a large amount of α-cellobiose octaacetate was separated in a crystalline form; yield 1.93 g. It was recrystallized twice from ethanol; m.p. and mixed m.p. 223–224°, $[\alpha]_D^{20}$ +39.4° (c, 1.9, chloroform); Calcd. for $C_{28}H_{38}O_{19}$: C, 49.56; H, H, 5.64; MW, 679. Found: C, 49.88; H, 5.71; MW, 740.

The mother liquor from the above crystallization of α-cellobiose octaacetate was re-chromatographed on "Magnesol-Celite" (5 : 1 by wt.) column, using benzene-*t*-butanol (100 : 1 by vol.) as the developer. Two zones appeared from the top of the column, 96–122 mm (A) and 138–156 mm (B) (other zones which appeared failed to produce crystalline material). Each of the zones was separated, and treated as described above. The acetate of zone A was crystallized from ethanol; yield 34 mg. The crystals were recrystallized from ethanol; m.p. 185–186°, $[\alpha]_D^5$, −18.7 (c, 1.1, chloroform), MW, 680. Zone B produced crystals from the ethanol solution; yield 42 mg. The material was recrystallized from ethanol; m.p. 187–188°, $[\alpha]_D^5$, +30.6° (c, 1.2, chloroform), MW, 640. Paperchromatography of the hydrolyzates of the two disaccharides revealed that they contained glucose at least as one component. The others were not confirmed.

Reversion Test

A mixture of 8.85 g of glucose, 0.85 g of mannose

[5] D.H. Brauns, *J. Am. Chem. Soc.*, **48**, 2776 (1926).

and 0.30 g of xylose was treated with the acetolysis reagent under the same conditions as which were used. The resultant white powder was deacetylated with 0.2 N-sodium methoxide and paperchromatographed. No oligosaccharides were detected on the paperchromatogram.

Acknowledgement. The authors wish to express their appreciation to Dr. H. S. Isbell, National Bureau of Standards, Washington, D. C., for generously supplying them with a sample of octaacetyl 4-D-glucosyl-β-D-mannose. They are also grateful to Dr. L. E. Wise, The Institute of Paper Chemistry, Appleton, Wisconsin, for kindly introducing them to Dr. H. S. Isbell, and Prof. K. Anno, Ochanomizu Univ., Tokyo, for her instruction extended in the techniques of "Magnesol" chromatography, and the Sanyo Pulp Co. Ltd., for contribution of dissolving pulp to the Institute.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 1, p. 14~16, 1958]

Isolation of a Diketopiperazine Derivative from the Soy-bean Meal Hydrolysates, Commercially Available as "AMINOSAN-EKI"

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Received September 7, 1957

A diketopiperazine derivative isolated from the soy-bean meal hydrolysates, AMINOSAN-EKI, has been proved as L-isoleucyl-L-valine anhydride by elemental analysis, melting point, and by paper chromatography. L-Isoleucyl-L-valine anhydride must be derived from the dipeptide produced by hydrolysis of the proteins.

INTRODUCTION

During an investigation of the sulfur compounds of the soybean meal hydrolysates, AMINOSAN-EKI, there was isolated a nitrogen compound which gave a negative ninhydrin reaction. This substance reacted with picric acid and *m*-dinitrobenzene[1], and it was therefore confirmed to be a diketopiperazine derivative which has been under discussion concerning its relation to protein structures.

EXPERIMENTAL

Isolating Procedures.

As shown in Fig. 1, commercially available AMINOSAN-EKI (18 l) prepared by acid (HCl) hydrolysis of soy-bean meal was concentrated in vacuo. The concentrates (6 kg) were extracted with 99% ethanol (50 l), and after removal of ethanol the residues (700 g) where consequently extracted with ethanol-ether 1 : 1 (20 l) by shaking. The solvent was evaporated in vacuo to a small volume and crystallized substances were obtained. The substances were first recrystallized from boiling absolute ethanol in which they were sparingly-soluble, and after several recrystallizations fine needles were obtained (0.5 g).

The crystal was found to sublime at about 160° and melt at 283–5° in a sealed tube. The substance did not react with ninhydrin and nitroprusside; it gave negative Millon, Folin, Sakaguchi, or Liebermann-Burchard reactions.

This crystal was almost insoluble in water and in dilute acid or alkaline, even on moderate heating. It was sparingly soluble in ethanol, acetone, or ethylacetate, and insoluble in ether. It dissolved in pyridine or glacial acetic acid easily.

As the result of the fusion of this compound with sodium, nitrogen was detected. It gave the typical color reaction with picric acid or *m*-dinitrobenzene which indicates the presence of a diketopiperazine structure.

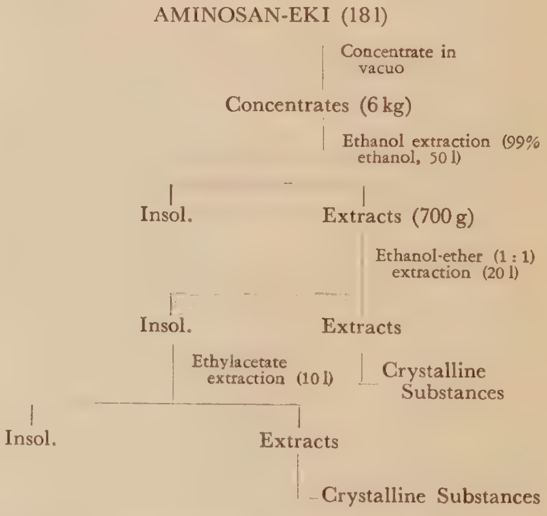

FIG. 1. Separation of a Diketopiperazine Derivative from Soy-bean Meal Hydrolysates.

By shaking extraction from the ethanol-ether (1 : 1) insoluble fraction, more crystals were obtained (0.3 g).

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[1] B. Suzuki and Y. Obata, "Biological Chemistry", Asakura-shoten, Tokyo, 1946, p. 493.

This procedure also yielded a same anhydride. Total anhydride (0.8 g) was recrystallized several times from 50% acetic acid. Fine needles (0.4 g), as shown in Fig. 2 were obtained, m.p. 308–310°.

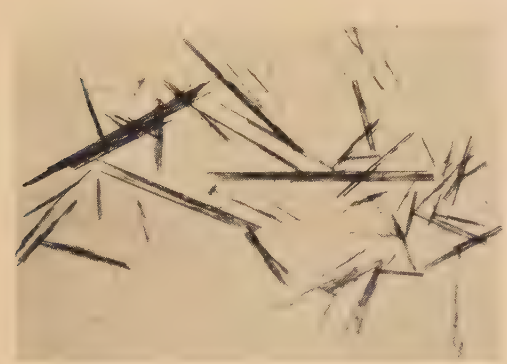


FIG. 2. Crystalline Diketopiperazine Derivative obtained from the Soy-bean Meal Hydrolysates, AMINOSAN-EKI.

This compound did not show any characteristic ultraviolet absorption.

Anal. Found: C, 62.41; H, 9.64; N, 12.77%. Calcd. for $C_{11}H_{20}O_2N_2$: C, 62.23; H, 9.50; N, 13.20%.

Hydrolysis of the Diketopiperazine Derivative.

The crystals (13.7 mg) were refluxed with 10 ml of 6 N HCl for 24 hours. Hydrolysate was neutralized with N NaOH, concentrated to a small volume in vacuo, and sodium chloride was removed by addition of ethanol.

The presence of L-isoleucine and L-valin was confirmed by paper chromatographical tests of this hydrolyzed mixture. These data are presented in Tables I and II.

TABLE I

COMPARISON OF HYDROLYZED DIKETOPIPERAZINE DERIVATIVE AND AUTHENTIC AMINO ACIDS BY PARALLEL PAPER CHROMATOGRAPHY IN A BUTANOL-ACETIC ACID-WATER SOLUTION (4 : 1 : 2)

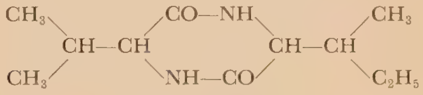
Amino Acid	R_F
Sample	0.66 ; 0.52
Norleucine	0.69
L-Leucine	0.67
L-Isoleucine	0.66
DL-Valine	0.52
α-Aminobutyric Acid	0.41

TABLE II

COMPARISON OF HYDROLYZED DIKETOPIPERAZINE DERIVATIVE AND AUTHENTIC AMINO ACIDS BY PARALLEL PAPER CHROMATOGRAPHY IN BUTANOL-ACETIC ACID-WATER (4 : 1 : 2)

Amino Acid	R_F
Sample	0.66 ; 0.52
Sample mixed with DL-Valine	0.66 ; 0.52
Sample mixed with L-Isoleucine	0.66 ; 0.52
Sample mixed with L-Leucine	0.67 ; 0.52
DL-Valine	0.52
L-Isoleucine	0.66
L-Leucine	0.68

Therefore, it is presumed the peptide anhydride is possibly a L-isoleucyl-L-valine anhydride, i.e., 1-isopropyl-4-*sec*-butylpiperazine-2,5-dione with the following structure:



DISCUSSION

L-Leucyl-L-valine anhydride[2] isolated from a casein-hydrolysate was reported, by E. Abderhalden and C. Funk, as having m.p. 282° (corr.).

L-Isoleucyl-L-valine anhydride[3] was isolated from hydrolyzed caseinogen by H. D. Dakin, and described to have m.p. 310–312° (uncorr.).

The former had been identified with synthetic L-leucyl-L-valine anhydride by mixture melting point determination. The latter had been hydrolyzed with hydrobromic acid, and good evidence had been obtained of the presence of both L-isoleucine and L-valine by examination of optical rotation in mixture, so that the original peptide anhydride had doubtlessly been a L-isoleucyl-L-valine anhydride.

The author's attention was next directed toward the mechanism of the formation of L-isoleucyl-L-valine anhydride during acid hydrolysis of protein. L-Leucine was used as a substitute for L-isoleucine. The equivalents of L-leucine and L-valine were refluxed with ten volumes of conc. hydrochloric acid for 48 hours, but anhydride was not detected. The addition of starch (a half-volume of two amino acids) yielded the same result.

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[2] E. Abderhalden und C. Funk, *Z. physiol Chem*, **53**, 19 (1907).

[3] H.D. Dakin, *Biochem. J.*, **12**, 314 (1918).

It is considered that the formation of anhydride will not be caused by condensation of two amino acid molecules, but by intramolecular condensation of a dipeptide which is an intermediate in the acid hydrolysis of protein. Such a peptide linkage consisting of amino acids having side chains as L-isoleucine and L-valine is inferred to be split with difficulty because of its steric hindrance.

Thus, when proteins were hydrolyzed with acid, comparatively stable dipeptides such as L-isoleucyl-L-valine remained, and it was considered that anhydrides may be produced by the action of acid. E. Abderhalden and C. Funk have proved that when L-leucyl-L-leucine was refluxed with five-volumes of 25% sulfuric acid for 16 hours, L-leucyl-L-leucine anhydride was formed.

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[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 1, p. 17~20, 1957]

Studies on the Essential Oils of Tobacco Leaves

Part XVI. Neutral Fraction (3)
Polycyclic Aromatic Hydrocarbons of Burley Tobacco Leaf

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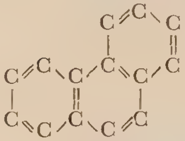
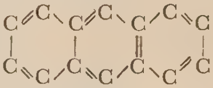
Received September 10, 1957

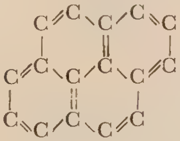
Phenanthrene, anthracene, pyrene and fluoranthene were isolated and identified from the neutral fraction of the essential oil of aged Japanese Burley tobacco leaf and their contents were determined.

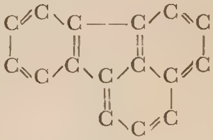
In the previous paper[1], the isolations of a saturated aliphatic hydrocarbon, which would be triacontane or hentriacontane, and another unsaturated aliphatic hydrocarbon resembling "Myrcene" separated from the neutral fraction in the essential oil of the aged Japanese Flue-cured tobacco leaf (JFCTL) were reported. Besides these, some fluorescent substances were observed on the chromatographic column during the isolating stage of these hydrocarbons, but they could not be identified because of their very small quantities. In this study, hydrocarbons in the neutral fraction of the essential oil of the aged Japanese Burley tobacco leaf (JBTL) were investigated and a large amount of fluorescent substances were obtained, from which several aromatic hydrocarbons were isolated.

After the acidic-, phenolic-, carbonyl- and basic-fractions were separated from the essential oil of JBTL by the same procedures as reported previously[2], the residual neutral fraction thus obtained was treated directly with 3-nitrophthalic anhydride, and free alcohols were separated as their half-esters. In order to separate oxygenated compounds from hydrocarbons, the alcoholic-free neutral fraction was chromatographed, using silicic acid as an adsorbent and *n*-hexane as the eluting solvent. Two strong fluorescent bands were observed on the early-stage of liquid-chromatography. Accordingly, four bands were separated by the help of these fluorescent bands. From the third- and forth-bands, phenanthrene, anthracene, pyrene and fluoranthene were separated and identified, and their contents were also determined.


Phenanthrene


Anthracene


Pyrene


Fluoranthene

On the other hand, from the first-band, paraffins and an unsaturated hydrocarbon resembling "Myrcene" were separated, as reported in the case of JFCTL, and a liquid aromatic compound was obtained from the second-band. Investigations upon these compounds are now proceeding.

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[1] I. Ōnishi, H. Tomita and T. Fukuzumi, This Bulletin, **20**, 61 (1956).

[2] I. Ōnishi and K. Yamasaki, This Bulletin, **19**, 137 (1955).

EXPERIMENTAL

(I) **Separation of Hydrocarbons:** The aged Japanese Burley tobacco leaf (124 kg) was steam distilled by the same procedures reported previously[2], and a dark-brown colored viscous oil (187,173 g., b.p. higher than 35°C) was obtained as the neutral fraction. The physicochemical properties of the neutral fraction of JBTL determined were as follows: d_{20}^{20} 0.9042; n_D^{20} 1.4810; $[\alpha]_D^{20}+5.73$; Saponification Value 37.52. Prior to any further procedures, the oil was treated with 3-nitrophthalic anhydride (equivalent in w/w) in an oil bath (100–115°) for three hours so as to separate the free alcohols. After the reacted product was allowed to cool, ether and water were added and extracted successively by shaking with an aqueous sodium carbonate solution (4%) in order to separate half-esters of the free alcohols and the unreacted reagent. The ethereal solution was dried over anhydrous sodium sulphate and the solvent was distilled off. In order to separate hydrocarbons from oxygenated compounds, the residual alcohol free fraction was chromatographed on a mixture of silicic acid and Celite (2:1 by weight) column, using *n*-hexane as an eluting solvent. Two bands, consisting of substances fluorescing under ultra-violet light as green-yellow and blue-green, were observed in the early stage of elution. Therefore, four bands were collected separately from the bottom band to the top one such as follows: (1) colorless band under ultra-violet light; (2) green-yellow fluorescent band; (3) very faint pale colored fluorescent band; (4) blue-green fluorescent band. The yields of each band are shown in Table 1.

TABLE I

SEPARATION OF HYDROCARBONS BY LIQUID-CHROMATOGRAPHY

Band Number	Yield (g)	Yield (mg/kg of leaf)	Property
H-(1)	10.641	85.8	Paraffins and unsaturated aliphatic hydrocarbons
H-(2)	0.964	3.7	Light-yellow liquid (aromatic hydrocarbon ?)
H-(3)	1.647	13.3	Light-yellow solid
H-(4)	0.465	7.8	Yellow-brown liquid
Total	13.717	110.6	

(II) **Separation and Identification of Phenanthrene, Pyrene and Anthracene:** The H-(3) band was purified by a successive liquid-chromatography, using a mixture of silicic acid and Celite (2:1 by weight) (column: 22×2.5 cm) as an adsorbent and *n*-heaxane as an eluting solvent. A yellowish-white solid matter (m.p. ca. 80–140°) was obtained after the eluting solvent was removed by distillation. An aliquot of this solid matter was purified again by a successive liquid-chromatography, using dry-packed alumina (column: 22×1.5 cm) as an adsorbent and cyclohexane as an eluting solvent. The eluted solution was measured directly by a Beckman Model spectrophotometer and phenanthrene, anthracene and pyrene were estimated by their ultra-violet spectra. Therefore, carbon bisulfide was applied to separate anthracene from other aromatic hydrocarbons by their solubilities[3].

Practically, the insoluble substance in carbon bisulfide was recrystallized three times from ethanol yielding white flakes, m. p. 205–208°[4]. The ultraviolet spectrum of this crystal showed maximum absorptions at 252, 324, 340, 357 and 376 mμ which were all identical with those of the authentic anthracene. The infrared spectrum of this crystal was also just identical with that of the authentic one. The mixed melting point with the authentic anthracene, m.p. 212°, showed no depression.

An aliquot of the easily soluble fraction by carbon bisulfide was recrystallized three times from ethanol yielding a white flaky crystal, m.p. 93–95°. The ultraviolet spectrum of this crystal has characteristic peaks at 251, 293, 274 and 281 mμ and infrared spectrum showed strong absorptions at 813, 734 and 715 cm^{-1} and both spectra were identical with those of the authentic phenanthrene (101°).

Another aliquot of the above mentioned carbon bisulfide readily soluble fraction was liquid-chromatographed on an alumina column[5] (21×2 cm.) by Wedgwood's method[6], using cyclohexane[7] as an developing solvent. The eluates, fractionated in every 20 ml were measured by a Beckman Model spectrophotometer and the fractions which indicated identical absorptions with pyrene were collected altogether. After distilling off the solvent, the eluates remained a white solid matter and was recrystallized from ethanol, white crystal, m.p. 147.3–148.1°. The mixed melting point with the authentic pyrene, m.p. 149–150°, showed no depression. The ultraviolet spectrum of this crystal agreed well with that of the authentic pyrene and the infrared spectrum was also identical with that of the authentic one.

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[3] Y. Yukawa, *J. Chem. Soc. Japan*, **63**, 147 (1942).

[4] All melting points mentioned in this paper were determined in sealed capillaries, and are not corrected.

[5] Merck's Reagent (for chromatographic adsorption analysis acc. to Brockmann.)

[6] Shaken with conc. sulfuric acid, several times until the acid solution became colorless, washed with water, dried over calcium chloride and distilled fractionally, b.p. 81°.

[7] P. Wedgwood and R. L. Cooper, *The Analyst*, **78**, 170 (1953).

(III) Isolation and Identification of Fluoranthene: An eluate from the H-(4) band had a yellow-orange viscous oil after removal of the solvent. Consequently, it was purified by a successive liquid-chromatography, using a mixture of silicic acid and Celite (2 : 1 by weight) (column : 20×2.5 cm.) as an adsorbent and *n*-hexane as an eluting solvent. The eluate corresponding to that of the H-(3) band, and other yellowish-brown colored matter retained heavily at the top of the column, were both rejected. After removal of the solvent from the middle principal effluent, yellow needles were obtained on cooling. However, as the crystal seemed to contain some oily contaminant it was purified again by a successive liquid-chromatography, using alumina column as an adsorbent and cyclohexane as an eluting solvent. A bluish-green colored band was separated under ultraviolet light from which light-yellow oil was obtained after removal of the solvent. Allowing to stand at room temperature yellowish-white needles were obtained and, after recrystallization two times from ethanol yellowish-white flatty needles, m.p. 108–110.2°, were obtained. The ultraviolet spectrum of these crystals in the ethanolic solution indicated maximum absorptions at 235 and 287 mμ which were identical with that of the authentic fluoranthene, and the infrared spectrum indicated strong absorptions at 827, 780 and 750 cm^{-1} which were also identical with those of the authentic one. The mixed melting point with the authentic fluoranthene, m.p. 110–111°, showed no depression.

TABLE II

QUANTITIES OF THE ISOLATED HYDROCARBONS

Constituents	Contents (mg) in 1 kg of leaf
Phenanthrene	5.00
Anthracene	4.21
Pyrene	1.80
Fluoranthene	1.38

On the other hand, the crystals were dissolved in hot ethanol and a saturated ethanolic solution of picric acid was added. Orange colored needles thus obtained were purified by recrystallization twice from ethanol. The mixed melting point of the purified crystals, m.p. 184.2°, with the picrate of authentic fluoranthene, m.p. 186°, showed no depression. The yields of these identified compounds are shown in Table II, which were not determined quantitatively but calculated from the weight of the isolated crystals.

DISCUSSION

The yield of the neutral fraction of the essential oil of JBTL exceeded that of JFCTL and the oil had a characteristic dark color. The specific tobacco-aromas were observed in both neutral fractions of JFCTL and JBTL. However, the neutral fraction of JBTL contains a somewhat fragrant odor caused by some aromatic compounds, besides the principal aroma of JBTL. The essential oil of JBTL comprises 7.3% of the hydrocarbons, but the content is less than that of JFCTL. However, on the contrary, aromatic hydrocarbons contained in the essential oil of JBTL are rather more abundant than those of JFCTL, which corresponds to the facts previously reported as follows: the essential oil of JBTL contains more phenols than that of JFCTL8); the essential oil of JBTL contains a very large amount of benzaldehyde in its carbonyl fraction9); benzoic acid was proved as a predominant component of the acid fraction of the essential oil of JBTL10) —these above-mentioned facts indicate that the essential oil of JBTL is rich in aromatic compounds.

All the polycyclic aromatic hydrocarbons, isolated and identified from the essential oil of JBTL in this study, have already been reported in tobacco smoke11, 12, 13) and it has been considered that they are composed of some pyrolitic products which is known to

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8) I. Ōnishi and K. Yamasaki, This Bulletin, **21**, 181 (1957).

9) I. Ōnishi and M. Nagasawa, *ibid.*, **21**, 95 (1957).

10) I. Ōnishi and K. Yamasaki, (may be published.)

11) B. T. Commins, R. L. Cooper and A. J. Lindsey, *Brit. J. Cancer*, **8**, 296 (1954).

12) R. L. Cooper and A. J. Lindsey, *ibid.*, **9**, 304 (1955).

13) J. Bonnet and S. Neukomm, *Helv. Chim. Acta.*, **39**, 1724 (1956).

occur in cigarette smoke[11,14]. It has also been reported that the smoke of cigarette-paper contains such polycyclic aromatic hydrocarbons[15].

Recently, Campbell and Lindsey[16] reported cyclohexane extractable polycyclic hydrocarbons in Virginia type cigarette and demonstrated that the amounts of them are very small. However, in this study these compounds were proved considerable amounts, especially, phenanthrene was found to be principal constituent which was not found in the above-mentioned cyclohexane extract. There is no presumption on the production of such tars obtained from tobacco leaf during the steam distillation process, as conducted in this study. On the other hand, these compounds could be isolated from the starting neutral fraction, prior to the separation of free alcohols by the use of 3-nitrophthalic anhydride. It seemed very interesting to note that polycyclic aromatic hydrocarbons must be contained in the aged tobacco leaf itself.

Among the various compounds isolated from the plant tissue, these polycyclic aromatic hydrocarbons are considered to be framework compounds having similar structures to some important plant components having significant biological relation. However, so far, no report has been found in the literature about the isolations of these compounds from plants. On the other hand, concerning the components of animals, it was reported that burnacles contain a series of these polycyclic aromatic hydrocarbons[17], but these compounds were estimated to be originated mainly from some tarry material floating in the water. Though we have no reasonable estimation in concern of the precursor of these compounds in tobacco plants, these compounds found in the tobacco smoke must be contributed not only to the components which were resulted by the tobacco burning, but also to the components of the essential oil of tobacco leaf.

Recently, the lung cancer problem seems to attract the attention of cigarette-smokers and several papers have been published concerning the interpretations of polycyclic aromatic hydrocarbons, probably contained in the tobacco smoke, and cancer producing causes. However, it is clear that these four compounds which were isolated and identified from the essential oil of tobacco leaf in this study, have been considered as non-carcinogenic substances[18,19].

Acknowledgement. We wish to express our cordial thanks to Prof. Y. Sumiki, Department of Agricultural Chemistry, University of Tokyo, for his wise direction and kind guidance, and to Director T. Nakashima, Central Research Institute, Japan Monopoly Corporation, for his strong support. We are also indebted to Miss K. Yamamoto, Mr. K. Saito, Mr. H. Takahara and Miss E. Sunakawa for their cooperation in carrying out this experiment. Last but not least, we are greatly indebted to Emeritus Prof. T. Yabuta for his sound advice.

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[14] J. B. Fishel and J. F. Haskins, *Ind. Eng. Chem.*, **41**, 1374 (1949).

[15] R. L. Cooper and A. J. Lindsey, *Chem. & Ind.*, 1260 (1954).

[16] J. M. Campbell and A. J. Lindsey, *Brit. J. Cancer*, **10**, 649 (1956).

[17] L. Zechmeister and B. K. Koe, *Arch. Biochem. Biophys.*, **35**, 1 (1952); *ibid.*, **41**, 396 (1952).

[18] A. Haddow, C. M. Scott and J. D. Scott, *Proc. Roy. Soc.* (London) **B122**, 477 (1937). *C. A.* **31**, 5446 (1937).

[19] E. C. Kooyman and J. W. Heringa, *Nature*, **170**, 661 (1952).

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 1, p. 21~23, 1958]

Chlorogenic Acid in the Tobacco Leaf during Flue-curing

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Received September 11, 1957

Chlorogenic acid in the tobacco leaf during flue-curing increases to about nine times as much as that of its content in the fresh leaf. This is shown as follows: fresh leaf, 0.34; warming stage, 0.71; streching stage, 0.68; yellowing stage, 1.61; fixing stage, 2.05; killing stage, 3.01; cured leaf, 3.06%. From above it is clear that chlorogenic acid in the tobacco leaf is formed in considerably great quantities during flue-curing.

Although polyphenols, mainly chlorogenic acid, in the tabacco leaf during flue-curing were discussed in detail by Frankenburg[1], little attention has been paid to the accurate content of chlorogenic acid during flue-curing. On the other hand, Porcsalmy[2] has found that tannin (polyphenols) in Hungarian types of tobacco leaves increases during normal air-curing and flue-curing, using the Löwenthal method to the determination of tannin. However, chlorogenic acid during flue-curing has not been determined till the present. In green or cured leaf, chlorogenic acid has been determined[3,4,5]. In this paper, after attempts were made, a paper chromatographic method similar to that described by Dawson and Wada[5] was adopted for the determination of chlorogenic acid during flue-curing. However, the chromatography, using the same filter paper and solvent, was almost impossible to give the same R_F values as those obtained by Dawson and Wada. This seemed to be due to the difference of the moisture of filter paper caused by humidity in the air, therefore, both the solvent and filter paper were exchanged with others.

EXPERIMENTAL

Plant Material: The adult leaves of Bright yellow (1957 crop) were supplied and flue-cured by the courtesy of the Utsunomiya Tobacco Experiment Station of the Japan Monopoly Corporation. The fourth and fifth leaves located from the top were primed and collected. The collected leaves were hung in a vertical position alternatively on straw-rope for flue-curing. The temperature and humidity during flue-curing are shown in Fig. 1. The times of sampling for investigation are shown in Table I. The leaves used for investigation were dried in a forced-draft hot-air oven at 70°C for one hour and ground by a mortar and stored.

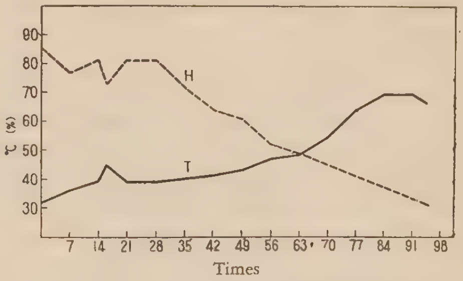

FIG. 1. Flue-curing Process.

H: Percent relative humidity inside the barn
T: Temperature inside the barn

Preparation of Extract: By a method similar to that described by Dawson and Wada, 10 g of dried powder was refluxed with 100 ml of 60 per cent methanol for thirty minutes on a boiling-water bath,

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[1] W.G. Frankenburg, *Advances in Enzymology*, **6**, 309-387 (1946).

[2] I. Porcsalmy, *Rev. intern. tabacs*, **28**, 223-5 (1953); *C.A.* **48**, 6656b.

[3] F.B. Wilkinson, M. Phillips, and A.M. Bacot, *J. Asso. Off. Agr. Chem.*, 1004-1012 (1954).

[4] M. Shiroya, T. Shiroya, and S. Hattori, *Physiol. Plant.*, **8**, 594-605 (1955).

[5] R.F. Dawson and E. Wada, *Tobacco Sci. pub. in Tobacco*, **144**, No. 11, 18-21 (1957).

Table I

Times and Temperatures of Sampling and Contents of Chlorogenic Acid

	Hrs.	Temp.	Chlorogenic acid	
			%[a]	ratio
I. Fresh leaf	0	32.0	0.34	100
II. Warming stage	12	36.0	0.71	208
III. Streching stage	19	39.0	0.68	200
IV. Yellowing stage	44	41.0	1.61	473
V. Fixing stage	61	47.5	2.05	602
VI. Killing stage	74	57.0	3.01	885
VII. Cured leaf	95	—	3.06	900

a: calculated to original dry-weight basis.
The calcium content in the leaf served as the basis for making corrections.

The extract was filtered and the residue was washed with the same solvent. The combined filtrate and washings were concentrated under reduced pressure, and transferred to a 50-ml volumetric flask and then made up to volume with water.

Quantitative Assay: Fifty microliters of the extract were applied to Tōyō No. 51 filter paper sheets (60×60 cm) and separated on the sheets with *n*-butanol-butylacetate-acetic acid-water (2 : 2 : 1 : 1) as the solvent by the descending method. After about six hours the strip was removed and allowed to dry in the air for one hour. Under these conditions, R_F values of chlorogenic acid showed 0.65–0.68. Depside B and C, described by Dawson and Wada, should give the same R_F values as chlorogenic acid obtained in this method. Consequently, the content of chlorogenic acid otained in this study, might have contained depside B and C. The strips cut out were eluted with water and the eluates were transferred to 10-ml volumetric flasks. Optical density measurements were made with a Beckman DU spectrophotometer at 324 mμ using the eluates from blank strips of the same filter paper for zeroing the instrument. The recovery of chlorogenic acid was 69±2%. The results obtained are shown in Table I.

RESULTS AND DISCUSSION

Roberts[6] described that if the leaf is still moist when the critical temperature is reached at which the semi-permeability of the protoplasmic membranes breaks down (about 55°C), polyphenols diffuse into the cytoplasm where they are oxidized to form brownish red pigments. According to his theory, the enzyme causing the oxidation of phenols and polyphenols is contained in the cytoplasm in the cells of the leaf, whereas the phenolic compounds are in the extracellular medium[7]. In flue-cured leaf the polyphenols are "frozen" in the leaf without further changes owing to the rapid removal of most of the moisture from the leaf and to the inactivation of the oxidative enzymes by the high final temperatures of the flue-curing process[8]. Therefore, it is assumed that the content of chlorogenic acid of fresh leaf should be rather more than that of flue-cured leaf. However, it is proved from the results of this investigation that the chlorogenic acid increases during flue-curing to about nine times as much as that content in the fresh leaf, especially more increases are observed in yellowing, fixing and killing stages, as shown in Table I. It is clear that the chlorogenic acid is formed in considerably great quantities during flue-curing, as Porcsalmy described about polyphenols in tobacco leaves during flue-curing[2].

The mechanism by which chlorogenic acid is formed during flue-curing is assumed to be as follows:

In the tobacco leaf during flue-curing, the enzymes, such as oxidase and peroxidase, are strongly activated[9,10] and the oxygen uptake increases[6,10]. As a result, oxidative respiration in the tissues comes to a high level. For the suppression of the Embden-Meyerhof-Parnas pathway by oxygen, known as the Pasteur effect, the direct oxidative pathway may take place exceedingly on the glycolytic process, as described by Engelhardt11). In consequence, chlorogenic acid may derive its benzene ring from a series of precursors through sedoheptulose-1, 7-diphosphate12,13), that is, a normal intermediate in the direct oxidative pathway, as that in the biosynthesis of the benzene ring of tyrosine, phenylalanine and tryptophane as described by Davis14).

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Acknowledgement. The author wishes to thank to Miss. Taeko Itaishi for her assistance in carrying out the determination of chlorogenic acid, and to Mr. M. Ihida, Mr. T. Kisaki, and Mr. K. Saito for their cooperations for sampling of the leaves.

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Studies on Amino Acids. X

Studies on the Enzymatic Resolution (VIII): Acylase Activity in *Eschericheae**

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The growth studies of methionine requiring mutant of *Escherichia coli* disclosed that several acyl methionines were utilized by the mutants. Furthermore, the cell-free extracts of *Escherichia coli* and *Aerobacter aerogenes* were found to show almost equal hydrolytic activity towards acyl amino acids.

In order to find the enzymatic properties of the acylase activity, the experiments were carried out with the alcohol-fractionated extracts of the acetone-dried cells of *Escherichia coli K-12*. As a result of the investigation, acylase activity of the preparation from *Escherichia coli* was found to be lower and also a lesser number of acyl amino acids were hydrolyzed than the mold acylase which had been previously studied by the authors. Activation by cobalt ion and several other observation were also presented.

Since several years ago, the authors have been studying on the acylase activity of molds, especially on its practical application for the optical resolution of amino acids[1～6].

Although a number of reports on the studies of acylase activity in animal and fungal sources have appeared, only a few reports have been published concerning the bacterial acylase. So, as a part of the studies aimed at obtaining complete knowledge on acylase activity occurring in various organisms, the authors have performed experiments on the acylase preparation from *Escherichiae*. The results obtained are presented in this paper.

On the acylase activity. that is, hydrolytic activity towards acyl amino acids, Imaizumi[7] reported that hippuric acid could not be hydrolized by the extracts of *Escherichia coli* but formyl-, acetyl-glycins and chloroacetyl-L-phenylalanine were hydrolized, although that last mentioned only slightly. Tomoda, Saito[8] and Kameda, Toyoura[9] also confirmed that the dried preparation and cell suspension of *Escherichia coli* could not hydrolize hippuric acid and benzoyl-DL-phenylalanine. During the course of authors' study, Vogel and Bonner[10] have reported on the acetylornithinase of *Escherichia coli* and have found that the acetylornithiase could deacetylate not only acetylornithine but also acetylmethionine. Although a number of other acyl amino acids had been tested, they were not susceptible to the preparation.

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As a preliminary test, the authors investigated the utilization of acyl methionines by *Escherichia coli.* Namely, several methionine requiring mutants of *Escherichia coli* were cultured on the basal medium with the addition of acyl methionines. As a result of this growth study, these acyl derivatives were found to be utilized by the mutants, which may be interpreted that the added acyl methionines were utilized for growth after being deacylated by the bacteria. In order to ascertain the presence of acylase activity, cell extracts of the parent strain of *Escherichia coli K-12* and of the mutant strain were studied and both extracts were found to be capable of hydrolizing acyl methionines. Furthermore, comparative study of the extracts of acetone-dried cells of *Escherichia coli* and *Aerobacter aerogenes* revealed that there was no difference of hydrolytic activity between both bacteria. Hence, to obtain information on the characteristics of this acylase system in *Escherichiae*, the following properties, i. e., hydrolytic activity towards various acyl amino acids, optimal pH, influence of metal ions and other enzymic properties were investigated by employing the alcohol-fractionated extracts obtained from acetone-dried cells of *Escherichia ocil K-12* as the enzyme preparation.

EXPERIMENTAL

Compounds Used The preparation of the amino acid derivatives was reported in the previous papers by the authors.

Organism The organisms used were *Aerobacter aerogenes*, *Escherichia coli K-12* and its methionine requiring mutants such as *58-161*, *Y-40* and *Ki-1023;* these were originated from the culture collection of the Department of Genetics, School of Medicine, Osaka University. The mutant strain *Ki-1023* was isolated by Kisumi, one of the authors, with UV radiation and subsequent penicillin screening[11].

Growth Study and Preliminary Tests The basal medium used for the growth study was the same as that employed by Davis[12]. To the test tube containing autoclaved basal medium, sterile-filtered solution of acetyl-, chloroacetyl-, glycyl-methionines and methionine were added in respective concentrations. One-loop of the appropriate microorganism which had been grown in a complete agar medium at 37° for 20 hours, was suspended in saline and then centrifuged. One drop of the saline suspension of the washed cells (ca 10^7 cells/ml), was used to inoculate each tube. Incubation was carried out at 37° for 20 hours, and growth was estimated by reading turbidities on a Beckmann model spectrophotometer at $515\ m\mu$. The result of this growth study is summarized in Table I. As shown in the Table, acetylmethionine was available for the growth of all mutants tested and the same growth was observed as in the case of methionine. Although chloroacetyl derivative was also fully available at lower levels, somewhat inferior growth was recognized at higher concentrations. Except for strain *Ki-1023*, replacability of glycylmethionine for methionine was very poor, especially in case of strain *Y-40* which could not grow with this glycyl derivative. It is worthy of note that although any difference of extent of utilization was not observed among the mutants tested in case of acyl derivatives, apparent difference between the mutant strains was observed in the case of the glycyl derivative.

TABLE I

UTILIZATION OF METHIONINE DERIVATIVES BY METHIONINELESS MUTANTS OF *ESCHERICHIA COLI*

Methionine derivs.	Concentration (M)	Growth of *E. coli**		
		58-161	*Y-40*	*Ki-1023*
none	—	99	99	99
L-Methionine	10^{-3}	59	59	59
〃	10^{-4}	59	60	59
〃	10^{-5}	82	83	81
Acetyl-L-methionine	10^{-3}	59	60	60
〃	5×10^{-4}	59	61	62
〃	10^{-4}	59	62	66
Chloroacetyl-L-methionine	10^{-3}	80	88	61
〃	5×10^{-4}	59	64	65
〃	10^{-4}	60	62	64
Glycyl-L-methionine	10^{-3}	88	99	61
〃	5×10^{-4}	84	99	61
〃	10^{-4}	82	99	61

* Expressed in terms of transmittance.

The above experiment infers that the acyl methionines were utilized after deacylation by an enzyme present in methionineless mutants of *Escherichia coli.* So, with the cell extracts of methionine requiring mutant *K-1023* and of its parent strain, hydrolytic activity to acyl- and glycyl-methionines was investigated. Namely, both strains were grown on a complete agar medium at 37° for 20 hours. The cells were harvested with M/30 phosphate buffer (pH 7.0) by centrifugation, suspended in the phosphate buffer, and collected again. Four grams of the wet cells were ground with quartz-sand and extracted with 40 ml of the phosphate buffer. The supernatant obtained by centrifugation was used as the extracts.

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As shown in Table II, although both parent and mutant strains were capable of hydrolizing acyl methionines, the activity of the former was found to be somewhat higher than the latter.

TABLE II

HYDROLYSIS OF METHIONINE DERIVATIVES BY CELL EXTRACTS OF *ESCHERICHIA COLI*

Methionine derivs.	Extent of hydrolysis (%) *K-12 wild*			
	4 hrs.	24 hrs.	*Ki-1023* 4 hrs.	24 hrs.
Acetyl-DL-methionine*	21	71	12	51
Chloroacetyl-L-methionine	9	47	5	30
Glycyl-L-methionine	100		100	

The incubation mixture contained 1 ml of 0.025 M substrate solution (0.05 M in the case of acetyl-DL-methionine), 1 ml of phosphate buffer and 1 ml of the extracts. The reaction mixture was incubated at 37° and pH 7.0. The liberated amino acid was measured by the colorimetric ninhydrin method.

* Extent of hydrolysis is expressed for the susceptible isomer of the substrate.

In order to compare the acylase activity of the above *Escherichia coli* and *Aerobacter aerogenes*, both strains were cultivated in the same way as described above, and the harvested cells were acetone-dried. The dried cells were extracted with M/30 phosphate buffer (pH 7.0) and centrifuged. The extracts were made up to a concentration corresponding to 5 mg of dried cells per ml. With these extracts, hydrolysis of various acyl amino acids was carried out at pH 7.0. The results, listed in Table III, indicate that although acyl derivatives of alanine, leucine, methionine and phenylalanine were hydrolyzed, the cleavage of the derivatives of glutamic acid, lysine, tryptophan and tyrosine did not occur under the conditions employed by the authors. Throughout the experiment almost no difference between both *Escherichеae* was observed, and it was also evident that the hydrolytic activity to glycylmethionine, a peptidase activity, was higher than the acylase activity in these crude extracts.

TABLE III

HYDROLYSIS OF ACYL AMINO ACIDS BY CELL-FREE EXTRACTS OF *ESCHERICHIA COLI* AND *AEROBACTER AEROGENES*

Substrate	Extent of hydrolysis (%) *E. coli*	 *A. aerogenes*
Clac-Alanine	9	11
Clac-Leucine	4	1
Ac-Methionine	56	49
Clac- 〃	20	20
Gly- 〃	82	36
Ac-Glutamic acid	0	0
Clac- 〃	2	1
ε-Bz-α-Ac-Lysine	0	0
ε-Bz-α-Clac- 〃	1	1
Ac-Tryptophan	0	0
Clac- 〃	0	0
Clac-Phenylalanine	19	2
Clac-Tyrosine	2	2

Ac: Acetyl, Clac: chloroacetyl, Gly: glycyl

The incubation mixture contained 1 ml of 0.025 M substrate solution (0.05 M for racemic compound), 1 ml of the extracts and 1 ml of M/15 phosphate buffer. The reaction mixture was incubated at 37° and pH 7.0 for 24 hours. The liberated amino acid was determined by the colorimetric ninhydrin method, and extent of reaction expressed as per cent hydrolysis of the susceptible substrate.

* Ten-times diluted extracts was employed.

Enzyme Preparation For the purpose to investigate the enzyme properties of acylase activity in *Escherichеae*, of which the occurrence of activity was ascertained through the experiments described above, a wild strain of *Escherichia coli K-12* was chosen for enzyme preparation. The cells grown on a complete medium were harvested, washed, and acetone-dried in the usual manner. The dried cells were extracted with 20-times of M/30 phosphate buffer (pH 7.0) and centrifuged. The supernatant solution was treated with alcohol (50% by volume) under chilling, the precipitate formed was removed by centrifugation and cold alcohol (70% by volume) was added to the supernatant solution. A precipitate formed, which contained 90% of the total activity of the crude extracts, was dissolved in phosphate buffer at pH 7.0. The resulting solution was employed as the stock enzyme solution, 1 ml of which corresponds to 25 mg of dried cells, and the activity assayed under the standard conditions described below by the use of acetyl-DL-methionine at pH 6.5, was 6.6 μM/hr/N mg protein. The stock solution kept by freezing, was thawed and properly diluted before it was used for the enzyme reaction.

Standard Enzyme Reaction Enzyme reaction was carried out by incubating digests composed of 1 ml of 0.025 M substrate solution (0.05 M for racemic compounds), 1 ml of enzyme solution and 1 ml of M/15 phosphate buffer (at appropriate pH, described in Table VI) at 37° for an appropriate period. Liberated amino acids during the incubation were measured by the colorimetric ninhydrin method, and expressed by per cent hydrolysis of the susceptible L-isomer.

For example, effect of enzyme concentration (varying dilutions of the stock enzyme solution) on the rate of hydrolysis under the standard conditions with acetyl-DL-methionine as the substrate, is shown in Fig. 1. The result indicated the extent of hydrolysis was proportional to enzyme concentration.

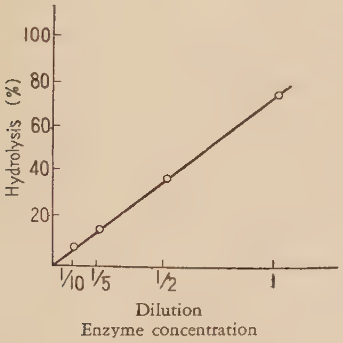

FIG. 1. Effect of Enzyme Concentration of Reaction Rate.

The mixture containing enzyme solutions of varying dilution and acetyl-DL-methionine was incubated at pH 6.5 for 3 hours under standard conditions.

Storage and Stabilization of the Enzyme Solution As for the above described enzyme preparation from *Escherichia coli* which lost activity during storage, stabilization of the enzyme solution was studied. Namely, glutathione, ascorbic acid, sodium sulfide and sodium cyanide were added as possible preservatives to the enzyme solution, and their activities were assayed after storage. Effect of storage by freezing was also investigated for comparison. As shown in Table IV, the stock enzyme lost about a half of its initial activity during the storage in a refrigerator at 5° for two weeks. The addition of ascorbic acid and sodium cyanide showed no preserving effect and sodium sulfide had ill-effect. Although the addition of glutathione was effective to prevent decrease of activity, it resulted in an undesirable increase of the enzyme blank in case of assay. So the most favorable step was to store the enzyme solution in the frozen state.

TABLE IV

EFFECT OF STORAGE TO THE ENZYME ACTIVITY

Exp. No.	Additions	Concentration*	Enzyme Activity (days)		
			3	7	14
1	None		75	70	47
2	None (freezing)		94	91	88
3	Glutathione	2×10^{-4} M	85	79	52
4	Glutathione	10^{-3} M	88	83	81
5	Ascorbic Acid	5×10^{-3} M	72	65	
6	Na_2S	10^{-3} M	15		
7	NaCN	//	69	60	

Enzyme assay was carried out under standard conditions for 2 hours with acetyl-DL-methionine. For the expression of enzyme activity, the activity of fresh preparation was taken as 100 and per cent activity was employed.

* Concentration in the enzyme solution.

As for the restoration of the diminished activity, the addition of cobalt chloride and glutathion to the fresh and deteriorated enzymes, was studied. The results, shown in Table V, revealed that the addition of cobalt and the simultaneous addition of glutathione with cobalt, stimulated the fresh enzyme activity in the same order of magnitude, while glutathione employed alone showed no stimulatory effect. In the case of stored enzyme, which activity had decreased to one-third of the initial activity, cobalt ion stimulated the remaining activity and reactivation of diminished activity could not be observed, although, the simultaneous addition of cobalt ion and glutathione was evidently effective for restoration.

TABLE V

RESTORATION OF THE DIMINISHED ENZYME ACTIVITY

Exp. No.	Addition (concentration)*	Storage (days)	
		0	16
1	None	100	36
2	Co^{++} (2×10^{-4} M)	309	130
3	Glutathione (10^{-3} M)	85	28
4	Co^{++} (2×10^{-4} M)+glutathione (10^{-3} M)	330	264
5	Co^{++} (2×10^{-4} M)+glutathione (3×10^{-4} M)	319	267

Enzyme assay was carried out under the same conditions as described in Table IV.

* Final concentration in reaction mixture.

Effect of pH In a previous paper⁶⁾, the authors

TABLE VI
OPTIMAL pH FOR THE HYDROLYSIS OF ACYL AMINO ACIDS

Acyl derivs.	Acetyl	Chloroacetyl
Alanine	6.2	5.9
Leucine	6.4	5.9
Methionine	6.5	5.6
Glutamic Acid	—	—
Aspartic Acid	6.7	7.3
ε-Benzoyllysine	—	5.1
Tryptophan	—	—
Phenylalanine	—	5.1
Tyrosine		—

The reaction was carried out under standard conditions for 6 hours.

observed that the optimum pH towards acetyl and chloroacetyl derivatives somewhat differed in the case of fungal acylase preparation. So, the effect of pH on the hydrolytic rate of various acyl amino acids by the preparation from *Escherichia coli* was studied and the optimal pH was determined. The results obtained are summarized in Table VI. Although the optimum pH for chloroacetyl derivatives seemed to be only slightly on the acidic-side than that of acetyl derivatives, no appreciable difference of optimal pH for both acyl derivatives as found in mold preparation could be detected. Among the chloroacetyl derivatives tested, optimal pH for that of aspartic acid was exceptionally on the alkaline-side. While, on the chloroacetyl derivatives of phenylalanine and ε-benzoyllysine, which the optimal pH existed on the acidic-side the experiments were repeated with Michaelis acetate buffer from which it was ascertained that there was no shift of the optimal pH in both buffers. Hydrolysis of the acyl derivatives of tryptophan and glutamic acid, acetyl derivatives of ε-benzoyllysine and phenylalanine, and chloroacetyltyrosine was not detected after prolonged incubation at pH 6.5.

Besides the acyl amino acids listed in the Table, optimum pH for glycylmethionine was also studied and it was estimated as 7.8.

Susceptibility of Acyl Amino Acids In the preliminary test, the extent of hydrolysis of typical acyl amino acids by the cell extracts of *Escherichia coli* and *Aerobacter aerogenes* at pH 7.0 was investigated. In this chapter, the susceptibility of the various acyl amino acids towards the bacterial acylase preparation was studied in the initial rates of hydrolysis at their respective optimal pH. The results obtained are summarized in Table VII, in which the rates of hydrolysis were converted to relative susceptibility taking that of acetyl methionine as 100 for the convenience of interpreting the results.

Acyl derivatives of monoaminomonocarboxylic acids such as alanine, leucine and methionine were hydrolyzed, especially, methionine derivatives were found to be most susceptible to the preparation. Further, hydrolytic activity to glycylmethionine, a peptidase action, appeared to be higher than the acylase activity. On the derivatives of monoaminodicarboxylic acids, although acyl glutamic acids were not hydrolyzed, chloroacetyl aspartic acid was found to be quite susceptible. While, among the lysine derivatives tested, only chloroacetyl derivative was hydrolyzed.

TABLE VII
RELATIVE SUSCEPTIBILITY OF ACYL AMINO ACIDS TO THE ENZYME PREPARATION OBTAINED FROM *E. COLI*.

Acyl Amino Acids	Susceptibility
Ac-Alanine	13
Clac- 〃	37
Ac-Leucine	3
Clac- 〃	12
Ac-Methionine	〔100〕*
Clac- 〃	87
Glyc- 〃	500
Ac-Glutamic acid	0
Clac- 〃	0
Ac-Aspartic acid	2
Clac- 〃	54
ε-Bz-α-Ac-Lysine	0
ε-Bz-α-Clac- 〃	32
Ac-Tryptophan	0
Clac- 〃	0
Ac-Phenylalanine	0
Clac- 〃	28
Clac-Tyrosine	0

Ac: acetyl, Clac: chloroacetyl, Glyc: glycyl, Bz: benzoyl

The enzyme reaction was carried out under the standard conditions for 6 hours.

* 6.6 μM/hr./N mg of protein

Generally, the acyl aromatic amino acids were less susceptible to this bacterial acylase preparation. Namely, except in case of chloroacetylphenylalanine, hydrolysis of the other acyl aromatic amino acids such as acetyltryptophan, acetylphenylalanine and chloroacetyltyrosine was not recognized under the conditions employed by the authors.

As in cases of acylase preparations obtained from other sources such as mold acylase and hog kidney

TABLE VIII

EFFECT OF METALS AND INHIBITORS ON THE ENZYME ACTIVIEY

Additions	Acetyl-methionine	Chloroacetyl-methionine	Chloroacetyl-phenylalanine	Glycyl-methionine
None	100	100	100	100
Cu	0	2	0	74
Mg	88	88	47	86
Ca	90	96	73	138
Zn	6	23	65	78
Mn	110	108	82	1100
Fe	103	107	120	128
Co	209	203	297	3800
Ni	11	39	53	97
EDTA	27	34	7	48
PCMB	6	22	3	7

The assay was carried out under standard conditions for 3 hours. Final concentration of the additions was 10^{-3}M. The activity of the respective substrate without addition was taken as 100. Metal chlorides were employed as metal ions in these experiments.

acylase, it was also observed in this bacterial preparation that chloroacetyl derivatives were more susceptible than acetyl derivatives. It is, however, of interest to note that exceptionally acetylmethionine was readily hydrolyzed than the chloroacetyl derivative.

Effect of Metals and Inhibitors In order to study the effect of metals and inhibitors, acetyl-, chloroacetyl-methionines, and chloroacetylphenylalanine were employed as the substrates. Various metal ions, ethylenediaminetetraacetate and *p*-chloromecuribenzoate were added at the enzyme assay and their activities were compared. Besides the above mentioned acyl amino acids, effect to the hydrolysis of glycylmethionine was also studied for comparison.

The results, shown in Table VIII, revealed that the effect towards the all acyl amino acids tested were almost the same. Namely, stimulation of acylase activity was observed with cobalt ion. Though salts of magnesium, calcium, manganese, and iron, at the concentrations tested, affected the reaction slightly; however, salts of copper, zinc and nickel acted inhibitory.

In the case of glycylmethionine, not only cobalt but manganese also stimulated the hydrolytic activity, and the order of mgnitude of activation with these ions was much higher than that of acylase activity. While, the inhibitory effect of salts of copper, zinc and nickel appeared to a far lesser extent.

Marked inhibition by metal binder, ethylenediaminetetraacetic acid (EDTA), and by the sulfhydryl reagent, *p*-chloromercuribenzoate (PCMB) were observed in all the substrates tested.

Along with the above experiment, stimulation by cobalt ion was evident, so the influence of the concentration of the ion was investigated by employing acetyl and glycyl derivatives of methionine as the substrates. Under the conditions employed, optimal concentration was found to be at 10^{-3} M, or 10^{-4} M, and a three fold activation in acylase activity and more than fortyfold stimulation of peptidase activity were observed as shown in Fig. 2.

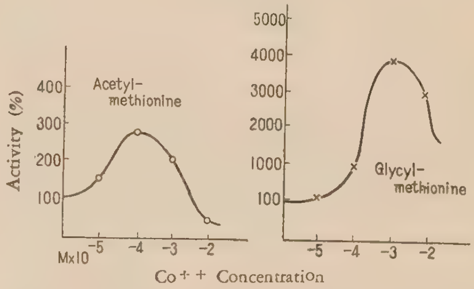

FIG. 2. Effect of Concentration of Cobalt on the Enzymatic Hydrolysis of Acetyl Methionine and Glycyl Methionine.

Experiments were carried out under standard conditions with the presence of varied concentrations of cobalt chloride for 3 hours.

Effect of Temperature For the purpose of studying the effect of temperature to the enzyme reaction, the extent of hydrolysis of acetyl-, chloroacetyl-, glycylmethionines and chloroacetyl aspartic acid as respective optimal pH and varied temperature was determined and is represented in Fig. 3. Maximal rates of hy-

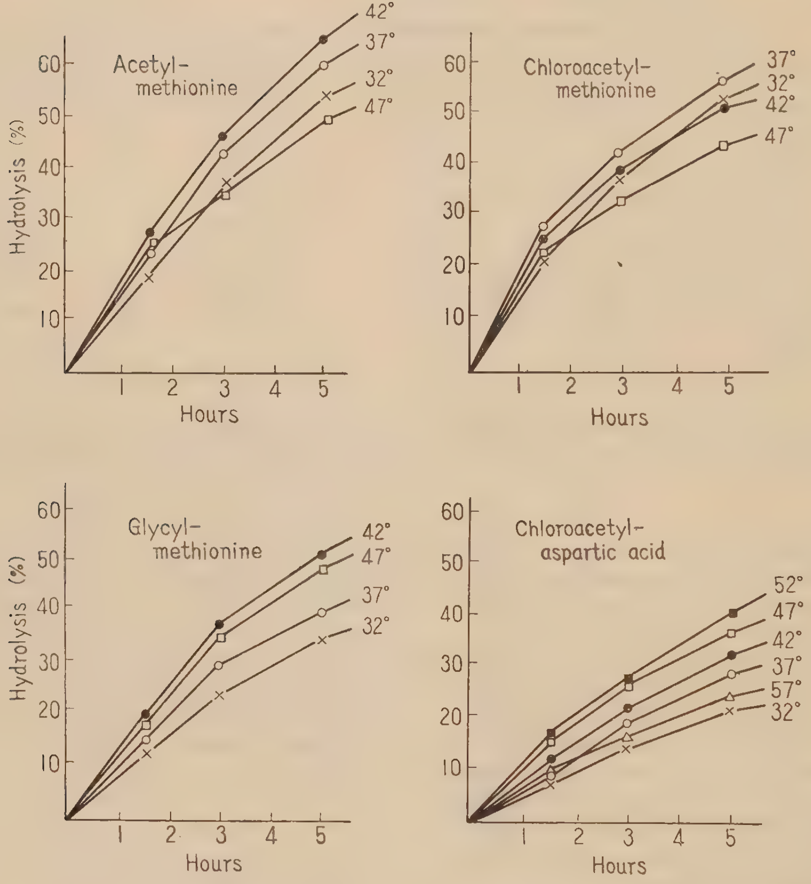

FIG. 3. Effect of Time and Temperature on the Hydrolysis by the Enzyme Preparation from *E. coli*.

The employed pH for the hydrolysis of acetyl-, chloroacetyl-, glycyl- methionines and chloroacetyl aspartic acid were 6.5, 5.5, 7.7 and 7.3, respectively.

drolysis were observed at 37° for chloroacetylmethionine and at 42° for acetyl, glycyl-methionines. While, the optimum temperature for the hydrolysis of chloroacetylaspartic acid was the highest, i.e., it was found to be at 52°.

Optical Specificity Since some of the bacterial enzymes had been known to act on the D-amino acid derivatives, optical specificity of this acylase preparation from *Escherichia coli* was investigated. The enzymatic reaction employing 0.025 M acetyl-L-methionine and 0.05 M acetyl-DL-methionine, as the substrate was simultaneously carried out under the standard conditions. As indicated in Fig. 4, in either cases, the extent of reaction stopped at the cleavage of 25 mM of the substrate, that is, 100% hydrolysis of L-form-substrate and 50% hydrolysis of racemic substrate. This result shows that the bacterial acylase preparation is optically specific and it acts asymmetrically on the L-form of acyl amino acid and not on the D-isomer. Whereas, it seemed that the presence of D-isomer did not appreciablly affect the rate of hydrolysis of the acyl-L-amino acid within the sensitivity of the method employed.

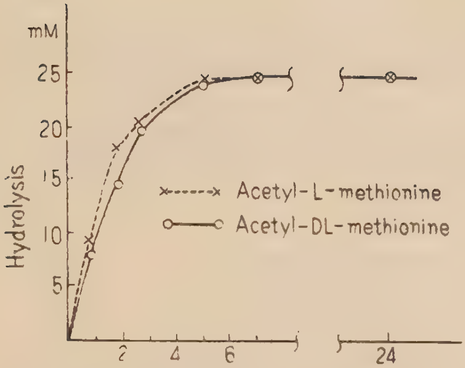

FIG. 4. Hydrolysis of acetyl derivatives of L- and DL-methionines by the enzyme preparation from *E. coli.*

The enzymatic reaction employing 0.025M acetyl-L-methionine and 0.05 M ,acetyl-DL-methionine was carried out at pH 6.5 under standard conditions. Extent of hydrolysis in the figure is expressed as mM of the liberated amino acid.

DISCUSSION

Through the growth study of mutant strains and the enzymatic study of cell extracts, the occurrence of acylase activity in *Eschericheae* has been ascertained.

Subsequent studies on the characteristics of the bacterial acylase preparation from *Escherichia coli* have revealed that although peptidase activity is high, acylase activity of this preparation is lower and the variety of the susceptible acyl amino acids is also less than that of previously studied fungal acylase preparation, which is capable to readily hydrolyze acyl derivatives of various types of aliphatic and aromatic amino acids. Especially, hydrolytic activity towards acyl aromatic amino acids appears to be very low, chloroacetylphenylalanine is the only susceptible one among those tested under the conditions employed by the authors.

The present study also reveals that this preparation resembles other acylase preparations such as hog kidney and mold acylases in several features. These features include the specific stimulation by cobalt ion. The inhibition observed by the addition of ethylenediaminetetraacetic acid and *p*-chloromercuribenzoate indicates that metal ion and sulfhydryl group play an important role in this enzyme action. Furthermore, optical specificity is also similar to other acylases, that is, L-form of the acyl amino acids is hydrolized selectively.

On the studies of optimum pH and temperature, somewhat exceptional behavior of chloroacetylaspartic acid was noted. Although the decision as to whether or not the difference observed is due to the presence of one or more specific acylases in this enzyme preparation, has to wait until the enzyme is obtained in further purified state.

In this study, attempts were not made to separate the aclyase activity from the peptidase action, although, the influence of metal ions towards both activities somewhat differed. For instance, peptidase activity is markedly stimulated not only by cobalt ion as in the case of acylase activity, but also by manganese ion. Also in another case, the inhibitory effect of copper and other metals observed in acylase activity, was not so evident.

As described above, some enzymatic properties of acylase activity in *Escherichiae* have been disclosed. The authors are intending to continue studies on the occurrence and enzymatic characteristics of acylase activity in various organisms in order to clarify biological significance of the presence of acylase activity.

Acknowledgements. The authors are grateful to Prof. H. Mitsuda of Kyoto University, and Dr. M. Fujisawa of this Laboratory for their helpful advice and encouragement. They were also indebted to Prof. H. Kikkawa of Osaka University for generously supplying the microorganisms used in this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 1, p. 32~34, 1957]

Studies on the Proteolytic Enzymes of Black *Aspergilli*

Part IV. Purification of Proteolytic Enzyme from *Aspergillus Saitoi* by Ion Exchange Resin

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Purification procedure of *Aspergillus Saitoi* proteinase was facilitated by the chromatography on a cation exchange resin (Duolite CS-101).

Introduction

In recent years a number of enzyme have been successfully chromatographed by elution analysis on columns of carboxylic exchange resin Amberlite IRC-50. These reports are chiefly related to the investigation of homogenuity of enzymes[1,2,3].

Chromatographical isolation of proteinase of *Bacillus subtilis* and that of *Streptomyces griceus* from the culture broth of the microorganisms have both been reported by Hagihara[4] and by Nomoto[5], respectively.

The ion exchange chromatography of enzymes, though its detailed theoretical explanation still remains obscure, is sure to be a highly useful technique for the isolation, purification, and examination of purity of enzymes.

We have previously isolated a crystalline proteolytic enzyme from *Aspergillus Saitoi* and have studied its enzymatic specificity[6,7,8].

In the present study, an investigation is described on the applicability of the ion exchange resin method to the purification of this enzyme under various conditions.

METHODS AND MATERIALS

a) Enzyme Preparation: The strain *Aspergillus Saitoi* (R-1149) was grown for 3 days at 30°C on wheat bran and the growth was extracted with three-folds of water. The extract thus obtained, was used in this experiment.

b) Ion Exchange Resin: Commercial Duolite CS-101 was made to Na-form with 1N-NaOH and pulverized with a ball mill. The resin which was passed through a 110-mesh screen was suspended in water, then made to H-form with 1N-HCl. After treated with acetone to dissolve impurities, the resin was washed with water to remove acetone and the resin was then bufferized with various buffer solutions (M/10).

c) Analyses of Effluent Fractions: For the determination of activity of proteinase and amylase, methods described in previous reports[6] were applied. Determination of protein concentration, was performed by use of the Copper-Folin method as described by Lowroy et al[9].

RESULTS AND DISCUSSION

A) Purification by the Batchwise-Method.

1) Adsorption of Proteinase and Amylase: One ml of enzyme extract was pipetted into

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[1] C. H. W. Hirs and S. Moore and W. H. Stein, *J. Biol. Chem.* **200**, 493 (1953).

[2] H. H. Tallan and W.H. Stein, *ibid.* **200**, 507 (1953).

[3] J. B. Neiland, *ibid.* **197**, 701 (1952).

[4] B. Hagihara, *Ann. Rept. Fac. Sci. Osaka Univ.* **2**, 35 (1954).

[5] M. Nomoto and K. Narahashi, Lecture delivered in July, 1956, at the Meeting of "Symposium on Enzymology".

[6] F. Yoshida, This Bulletin, **20**, 254 (1956).

[7] F. Yoshida and M. Nagasawa, *ibid* **20**, 257 (1956).

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[9] O. H. Lowry, N. J. Rosenbough, A.L. Forr and R. T. Randall. *J. Biol. Chem.*, **193**, 265 (1951).

a test tube containing 2 g of Duolite Cs-101 which have been previously bufferized with various buffers in the pH range 2.0–4.5 and the total volume was adjusted to 5 ml by the addition of buffer after which the mixture was centrifuged and the supernatant was used to determine enzymatic activity. The result is shown in Fig. 1.

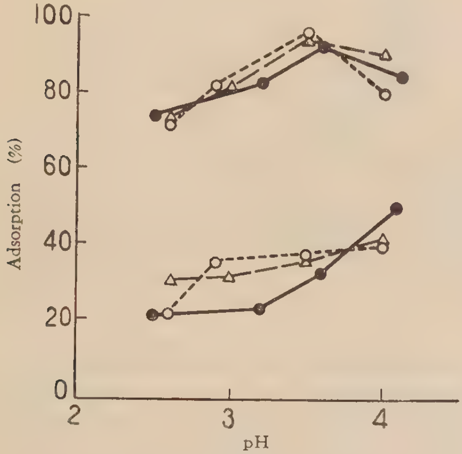

FIG. 1. Adsorption of Proteolytic Enzyme and Amylolytic Enzyme of *Asp. Saitoi* on Duolite CS-101 with Various Buffers.

-○----○- McIlvaine buffer
-●—●- $\frac{\mathrm{M}}{10}$ acetate buffer
-△—△- $\frac{\mathrm{M}}{10}$ tartarate buffer

It is recognized that there is a remarkable difference in the distribution coefficient between proteinase and amylase. Thus, it was decided to adsorb proteinase by the use of M/10 acetate buffer (pH 3.5).

2) Elution of Proteinase: The resin which adsorbed proteinase was eluted by various buffers in the pH range of 4.0–6.0. As shown in Fig. 2, the proteinase could be eluted either by M/5 acetate buffer (above pH 5.0) or MacIlvaine buffer (above pH 6.0). However, since this enzyme is more stable at pH 5.0 than at pH 6.0, it is favourable to elute with acetate buffer (pH 5.0).

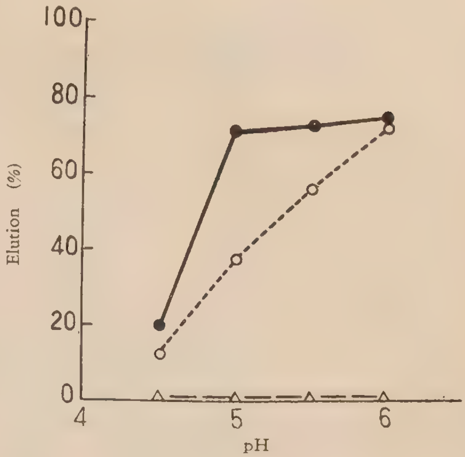

FIG. 2. Elution of Proteolytic Enzyme of *Asp. Saitoi* from Duolite CS-101 with Various Buffers.

-○----○- $\frac{\mathrm{M}}{2}$ McIlvaine buffer
-●—●- $\frac{\mathrm{M}}{2}$ acetate buffer
-△—△- $\frac{\mathrm{M}}{2}$ phosphate buffer

TABLE I

SUMMARIZED DATA OF PURIFICATION OF *Asp. Saitoi* PROTEINASE BY THE BATCHWISE METHOD

	Proteinase	Amylase
Initial Solution	100%	100%
Treated with Duolite CS-101	19.9	60.3
Washing Water	5.4	22.5
Retained in Duolite CS-101	74.7	17.2
Eluted with Acetate Buffer (pH 5.1)	74.0	5.3

Table I. shows summarized data of the purification of *Asp. Saitoi* proteinase conducted by the batchwise method.

B) Column Chromatography on Preparative Scale.

The apparatus which was appropriately designed for the use of a 3×70 cm column of resin was employed here. The column was bufferized at pH 3.5 with M/10 Na-acetate-HCl buffer and then 100 ml of enzyme solution (pH 3.5) was added to the column and it was then washed with dilute HCl (pH 3.5).

The flow-rate was 30 ml per hour and fractions, each 10 ml were collected. After removal of the amylase, M/2 acetate buffer

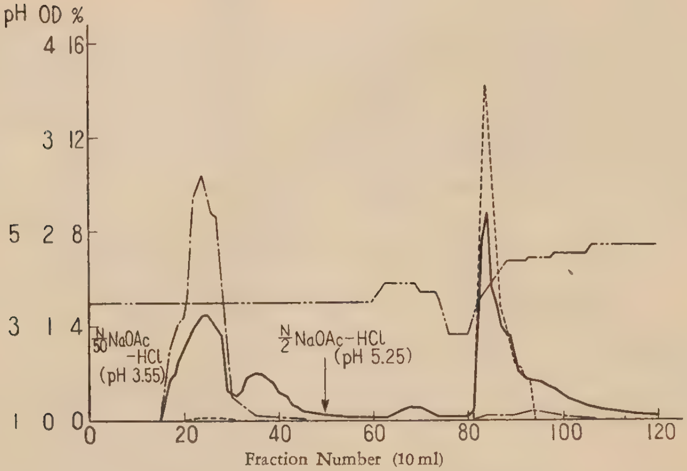

FIG. 3. Chromatographic Separation of Proteolytic Enzyme and Amylolytic Enzyme of *Asp. Saitoi* with Duolite CS-101.

———	protein	—·—·—	amylolytic enzyme
---------	proteolytic enzyme	—··—	pH

(pH 5.2) was added to the column and the proteinase was eluted with a yield of 60–70%. The enzyme activity of this fraction (fraction number 80–90) was about 8 times as strong as that of the initial solution. Though this fraction still contained a considerable amount of impurities, the proteinase could be easily crystallised in high yields by application of the previous method. Fig. 3 and Table II show the purification procedure by column chromatography and the yield of proteinase at each stage, respectively.

TABLE II

YIELD AND ACTIVITY DURING THE PURIFICATION PROCEDURE OF *Asp. Saitoi* PROTEINASE

Preparation	Yield	Activity PU/PN mg
Initial Solution	100%	330
Proteinase Fraction Eluted from the Column	63	2500
$(NH_4)_2SO_4$ Salting-Out	52	3200
Acetone Precipitated Solution	35	5500
Crystal	11	6750

From the results obtained here the purification of *Aspergillus Saitoi* proteinase was facilitated without complicated treatment as described in our previous report⁶⁾. In the purification procedure the use of other resins such as Amberlite XE-64 was also found practicable under the same condition.

SUMMARY

The Purification of *Aspergillus Saitoi* proteinase by the use of ion exchange resin (Duolite CS-101) was studied.

Aspergillus Saitoi proteinase can be purified on the column of Duolite CS-101. This method also permitted the separation of proteinase and amylase from crude enzyme preparations.

We wish to express our thanks to Prof. K. Sakaguchi, Prof. T. Asai for their kind guidance and also to Dr. M. Mogi for his support.

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Purification of Alkaline Proteinase of *Aspergillus sojae* by Ion Exchange Resin

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A method for the purification of the alkaline proteinase of *Aspergillus sojae* by the use of a carboxylic acid ion exchange resin, Amberite IRC-50 (XE-64) was studied.

The alkaline proteinase of *Aspergillus oryzae* which shows maximal activity at pH on the alkaline-side has been purified and crystallized by Crewther et al.1), Akabori et al.2) and Miura3). The key point in the purification procedures is the removal of amylase from crude enzyme. For this purpose, either differential fractionation of proteinase and amylase with ethanol, or starch adsorption, or rivanol precipitation of amylase has been employed. For purification of proteinases of *Bacillus subtilis*4) and *Streptomyces griseus*5), phenol-formalin ion exchange resins are usable.

Enzymes which can be purified by ion exchange resins include cytochrome c6,7,8), ribonuclease9), lysozyme10) and chymotrypsinogen11,12). In this laboratory, the authors have found that removal of amylase from crude enzyme extracts of *Aspergillus sojae* could be performed by means of Amberite IRC-50 (XE-64) and have now made that purification and crystallization of proteinase of this mold have considerably easy.

MATERIALS AND METHOD

Ion Exchange Resin Amberite IRC-50 (XE-64) was used. The resin suspended in water was decanted to remove the extremely fine granules, and the rest in suitable fines were treated with sodium hydroxide followed by with hydrochloric acid in the manner described by Hirs et al.9). The resin, usually in the powder of 200 to 400 mesh, was equilibrated with N/20 acetate buffer.

Preparation of Mold Enzyme *Aspergillus sojae* K. S. was cultured for three days at 30°C on wheat bran and the bran cultures was then extracted with two or three folds of water. The mold extracts were used for the study. In some cases, enzyme preparations which had been precipitated by ethanol and then by acetone were used in the experiments.

Estimation of Enzyme Activity and other Analytical Method The proteinase activity was measured by a modification of the Anson method13) at the optimal pH 10.0. The saccharifying and liquifying amylase activities were estimated by the amount of reducing sugar formed and by iodine starch reactions, respectively. The quality of protein in enzyme solution was determined by a modification of the Lowry method14). The quantity of pigments was estimated colorimetrically.

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1) W. G. Crewther and F. G. Lennox, *Aust. J. Biol. Sci.*, **6**, 428 (1953).

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3) Y. Miura, *Ann. Rev. Takamine Lab.*, **6**, 14 (1955).

4) B. Hagihara, *Ann. Rep. Fac. Sci. Osaka Univ.*, **2**, 35 (1955).

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10) H. H. Tallan and W. H. Stein, *J. Biol. Chem.*, **194**, 793 (1952).

11) C. H. W. Hirs, *J. Biol. Chem.*, **205**, 93 (1953).

12) N. Sakoda, S. Kubo, Y. Okuda and S. Akabori, Symposia on Enzyme Chem., **10**, 155 (1954).

13) M.L. Anson, *J. Gen. Physiol.*, **22**, 79 (1938).

14) O. H. Lowry, N. J. Rosenbrough, A. L. Forr and R. T. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

RESULTS AND DISCUSSION

A. Preliminary Experiments

1) Relationship between pH of buffered resin and adsorption of proteinase and amylase: In the preliminary experiments, the enzyme preparation which were extracted from mold culture and precipitated by ethanol and by acetone were employed.

Ion exchange resin XE-64 (1.8 ml) was buffered at several different pH values with N/20 acetate buffer, and 1 ml of the enzyme solution (20 mg protein/10 ml) and 5 ml of acetate buffer (N/20) of each pH were added to the resin. After mixing for ten minutes, the mixture was centrifuged (8000 r.p.m. 5 min. at 0°C). Activities of proteinase and amylases of the supernatant fluid were assayed resulting as shown in Fig. 1.

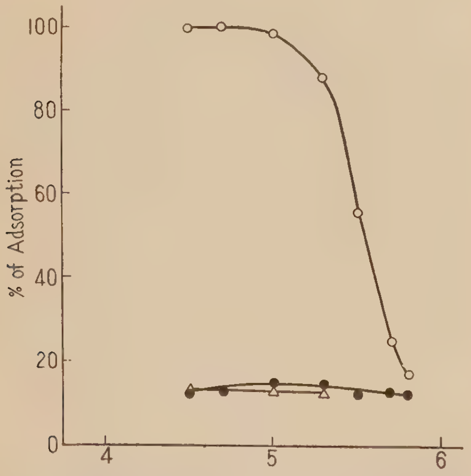

FIG. 1. pH-Adsorption Curve of Crude Enzymes of *Aspergillus sojae* on XE-64.

—○——○— Proteinase
—●——●— Saccharifying Amylase
—△——△— Liquifying Amylase

Considerable differences in distribution of proteinase and amylases in the resin-solvent system were observed. In Fig. 1, it is seen that almost all the proteinase in the enzyme solution is adsorpted on the resin below pH 5.0, while a small portion (13 to 15%) of the saccharifying and liquifying amylases is adosorpted on it under this condition. It was also found that Duolite CS-101 can be used similar to Amberite IRC-50 for purification of this mold proteinase.

2) Elution: The resin XE-64, on which proteinase was adsorpted at pH 5.0, was washed and centrifuged three times with 5 ml of N/100 acetate buffer (pH 5.0) in order to remove amylases and other impurities adsorpted. During this procedure, only a negligible amount of proteinase was lost. Subsequently, proteinase was eluted with 5 ml of various kinds of buffer solutions (pH 7.0, M/5) resulting in a yield above 80% in all cases. In the eluate, only 3 to 5% of amylases, about 2% of neutral proteinase[15] whose optimal pH lies in the range between pH 5.0 and 6.0, and a small amount of pigments were contained. (Table I)

TABLE I
YIELDS OF ENZYME CONTAINED IN ELUATE

Buffer M/5, pH 7.0	Proteinase	Amylase	Neutral Proteinase
Citrate-phosphate	89.9%	3.3%	1.4%
KH_2PO_4-Na_2HPO_4	89.9	2.4	—
Phosphate-borate	80.8	4.9	—
Phosphate-NaOH	88.4	2.4	—

Even when mold bran extracts are used in place of enzyme preparation precipitated by ethanol and by acetone, almost the same results were obtained.

From the above experiments and from the fact that *Aspergillus sojae* K. S. produces a far less quantity of acid[16] and neutral proteinase than that of alkaline proteinase, it is easily expected that Amberite IRC-50 (XE-64) can be successfully employed for the purification of the alkaline proteinase.

An example of purification experiments is described in the next section.

B. Purification by the Batchwise Method

The enzyme solution extracted from bran culture with three folds of water and adjusted to pH 4.8 by hydrochloric acid was immediately treated at lower temperatures throughout all the processes. (Fig. 2)

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[15] T. Yasui, Lecture delivered at the Agr. Chem. Soc. meeting on Jan. 1955.

[16] N. Iguchi and K. Yamamoto, *J. Agr. Chem. Soc. Japan*, **29**, 387 (1955).

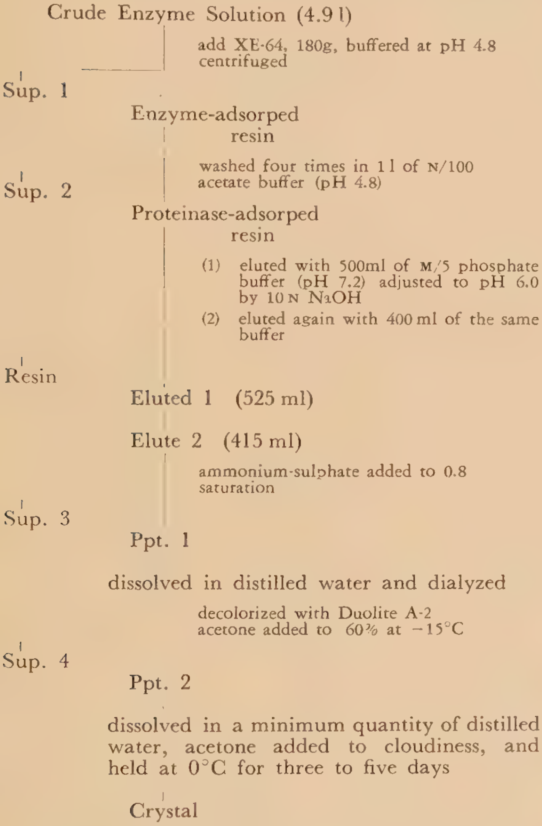
Crude Enzyme Solution (4.9 l)
add XE-64, 180g, buffered at pH 4.8
centrifuged
Sup. 1
Enzyme-adsorped resin
washed four times in 1 l of N/100 acetate buffer (pH 4.8)
Sup. 2
Proteinase-adsorped resin
(1) eluted with 500ml of M/5 phosphate buffer (pH 7.2) adjusted to pH 6.0 by 10 N NaOH
(2) eluted again with 400 ml of the same buffer
Resin
Eluted 1 (525 ml)
Elute 2 (415 ml)
ammonium-sulphate added to 0.8 saturation
Sup. 3
Ppt. 1
dissolved in distilled water and dialyzed
decolorized with Duolite A-2
acetone added to 60% at −15°C
Sup. 4
Ppt. 2
dissolved in a minimum quantity of distilled water, acetone added to cloudiness, and held at 0°C for three to five days
Crystal

FIG. 2. Purification Procedure of *Aspergillus sojae* by IRC-50 (XE-64).

TABLE II. YIELDS AND ACTIVITY OF EACH FRACTION OF *ASPERGILLUS SOJAE* PROTEINASE AND AMYLASE

	Proteinase	Amylase	PU/mg Protein-N
Enzyme solution	100%	100%	700
Supernatant 1	7.3	81.6	—
Elute 1	45.7	0.54	3800
Elute 2	16.3	0.38	3600
Ammonium-sulphate salting out	47.4	—	4500
Crystal	5.0	—	7200

For effective removal of amylases, it is necessary to wash the enzymes adsorpted to resin repeatedly with dilute buffer (N/100, pH 4.8). The recovery percentage of proteinase in the eluates was from 62 to 65%, while that of amylases was below 1% in total (Table II).

As the volume of the eluate fairly increased, ammonium-sulphate precipitation was carried out before further purification.

The proteinase preparation thus obtained was accompanied by some amounts of pigments, but almost all of the pigments could be removed by Duolite A-2.

The purity of proteinase in eluate was 50 to 53% as compared with crystalline proteinase. The proteinase preparation obtained by further ammonium sulphate salting-out showed about 62% purity of crystalline proteinase. Crystallization of proteinase from the proteinase preparation can be carried out without much difficulty. (Fig. 3)

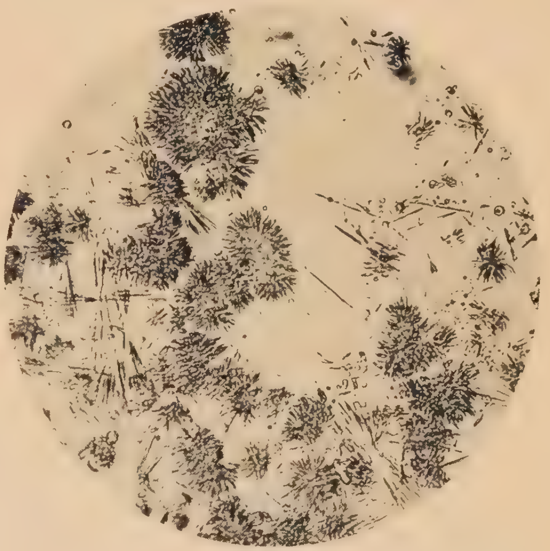

FIG. 3. Microphotograph showing Crystalline Alkaline Proteinase obtained from *Asp. sojae* (×1200).

C. Chromatography

A 2×32 cm column was prepared with Amberite IRC-50 (100 to 200 mesh) which was previously buffered with acetate buffer at pH 4.8. One hundred-ml of mold bran extracts adjusted to pH 5.0 by hydrochloric acid was chromatographed around 10°C. In the present experiments, the column was sufficiently washed with dilute acetate buffer (N/100, pH 5.0) before elution of proteinase.

The eluent was 0.2 M phosphate buffer of pH 7.0. The effluent fractions were 15 ml in volume and were collected at the rate of two fractions per hour. In the case of fraction numbers 127 to 134, however, they were 10 ml and three fractions, respectively. Determination of enzymic activity and estimation of protein and pigments were performed on aliquots removed from each of the tubes.

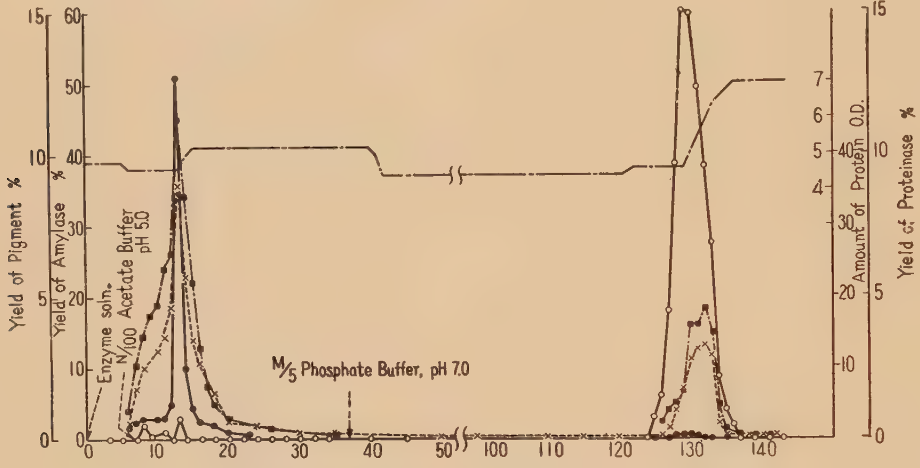

FIG. 4. Chromatography of Alkaline Proteinase of *Aspergillus sojae*

—○——○— Proteinase
--×---×-- Protein
——●—— pH
—●——●— Amylase
—■——■— Pigment

In Fig. 4, are given the results obtained. The first peak consists of almost all of the amylases (99%) and a trace of proteinase (1 to 2%). About 70% of pigments of the original enzyme solution are contained in this peak. The second peak contains about 80% of proteinase and small amount (2 to 3%) of amylases. The proteinase peak, however, is accompanied with apprx. 23% of pigments.

The quantity of proteinase available for the further purification was nearly 69% in the six fractions (fraction numbers 128 to 133, volume 60 ml), and that of amylases and of pigments in these fractions were about 2% and 19%, respectively. Almost all the neutral proteinase was also detected in the amylase peak.

SUMMARY

The alkaline proteinase of *Aspergillus sojae* is nearly completely adsorpted on Amberite IRC-50 (XE-64) buffered at pH 4.8 to 5.0, while amylases and other impurities are adsorpted to a less amount. Purification by batchwise method is described.

After removal of impurities, yield of proteinase and that of amylases in the eluate were 62% and 1%, respectively. The purity of proteinase in the eluates as compared with crystalline proteinase was from 50 to 53%. After ammonium sulphate salting-out it was apprx. 62%.

Chromatographic analysis was carried out with the same results obtained.

The authors wish to express their gratitude to Professor K. Sakaguchi and Professor T. Asai for their guidance, and are also greatly indebted to Mr. F. Yoshida for his valuable advice and criticism.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 1, p. 39~46, 1957]

Formation of L-Glutamic Acid from γ-Aminobutyric Acid by Plant Enzyme

Part I. Interconversion of α-Ketoglutaric-γ-Aminobutyric Transaminase and L-Glutamic Acid Decarboxylase Preparation in Plants*

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Received September 30, 1957

In the course of studies concerning the reverse action of L-glutamic acid decarboxylase, the authors have asserted the inhibition of α-ketoglutaric acid and the presence of L-glutamic acid and γ-aminobutyric acid in the enzyme preparations. Finally, evidence for the interconversion of α-ketoglutaric-γ-aminobutyric transaminase and L-glutamic acid decarboxylase preparations isolated from plant tissue was established in our laboratory.

INTRODUCTION

A number of reports have already appeared showing that there are many metabolic pathways for L-glutamic acid synthesis in organisms. Recently, studies on the fermentative production of L-glutamic acid are in rapid progress in Japan. H. Katagiri[1] et al. have reported on the α-ketoglutaric acid fermentation by *Coli aerogenes* bacteria in which a large amount of α-ketoglutaric acid obtained was converted to L-glutamic acid by the enzymic transamination. Thenceforth, K. Sakaguchi[2] et al., succeeded in fermentative production of L-glutamic acid from α-ketoglutaric acid and ammonium salt, and also by S. Kinoshita[3] et al., in establishing a method for the production of this substance from glucose. T. Asai[4] et al. also reported about the L-glutamic acid production from glucose and ammonium salt by a strain of *Micrococcus varians*.

Through the efforts of many investigators, it is now apparent that a number of metabolic pathways of L-glutamic acid are to be catalyzed by enzyme preparations of animal tissues and microorganisms, but rather a few of them in plant tissues. In 1937, K. Okunuki[5] first discovered L-glutamic acid decarboxylase in higher plants which he afterwards succeeded to isolate. In 1955[6], the same author studied on the metabolism of γ-aminobutyric acid by means of bacteria suspension, and confirmed that the enzyme system metabolizing γ-aminobutyric acid requires Fe^{++}. In 1954, Martin[7] et al., studied on the enzymic synthesis of

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* Presented at the meeting of the Agricultural Chemical Society of Japan, Tokyo, September 28, 1957.

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[7] E.H. Martin et al., Division of Biological chemistry. C & EN (1954).

L-glutamic acid from γ-aminobutyric acid plus CO_2 by the action of L-glutamic acid decarboxylase, and the equilibrium constant for the decarboxylation of glutamate $K=(\gamma\text{-aminobutyrate})(HCO_3^-)/(\text{glutamate}^-)=70$ was determined by treatment with L-glutamic acid decarboxylase in the presence of $C^{14}O_2$. In 1953, J. K. Miettinen and A. I. Virtanen[8] studied on the presence of transaminase in a suspension composed of α-ketoglutaric and γ-aminobutyric acids participating in the normal metabolic cycle containing L-glutamic acid in plants. In 1956, F. C. Steward[9] et al., also, investigated the role of γ-aminobutyric acid in relation to glutamine, using tissue slices of higher plants, and considerded γ-aminobutyric acid to be an important metabolite for growing and non-growing plant cells.

Quite recently, in 1957, O. Schales and S. S. Schales[10] studied on decarboxylations and transaminations in extracts and dry powder of higher plants, and reported that many plant extracts contained an enzyme system which liberates 2–3 moles of CO_2 with α-ketoglutaric acid as the substrate in addition to L-glutamic acid decarboxylase and α-carboxylase. From this fact, the same authors suggested that the reaction of plant extracts with α-ketoglutaric acid may be a transamination process leading to the formation of L-glutamic acid in plants, but it was not identificaly demonstrated in their data.

From all the studies referred to the above, experiments seem to have been performed with cell suspension or tissue slice, but not with cell-free enzyme preparation, except in the case of L-glutamic acid decarboxylase as described by Okunuki.

From our particular interest on this subject, an attempts have been made to demonstrate the reverse reaction of L-glutamic acid decarboxylase in order to elucidate whether or not the purified enzyme preparation can produce L-glutamic acid from γ-aminobutyric acid.

In 1954, the present authors[11] published the results of studies on the distribution and activities of L-glutamic acid decarboxylase from 50 kinds of edible plants.

Afterwards, our co-workers[12] continued investigations, and the effect of various factors on the activity of the enzyme was accurately tested. The enzyme action was always confirmed to be increased by pyridoxal-phosphate as it is already known, which on the contrary, it is severely inhibited by α-ketoglutaric acid. During these experiments, our co-workers confirmed the occurrence of L-glutamic acid and γ-aminobutyric acid in the same tissue containing L-glutamic acid decarboxylase. From these findings, it has been suggested that α-ketoglutaric-γ-aminobutyric transaminase may be detected in the same plant tissue containing L-glutamic acic decarboxylase, and transaminase activity was determined as markedly positive in the transparent fluid freshly prepared from many kinds of plant tissue described in the previous paper.

Finally, further evidence was established for α-ketoglutaric-γ-aminobutyric transaminase activity in the L-glutamic acid decarboxylase preparation obtained according to scheme 2, and simultaneously dry transaminase preparation was also isolated in a cell-free state from cold transparent plant fluid by partial saturation with ammonium sulfate.

γ-Aminobutryric acid was freshly prepared from synthetic 2-pyrrolidone, and α-ketoglutaric acid, produced by the fermentative method according to H. Katagiri[1], was supplied by the Ajinomoto Co. Inc.. After repeated investigation, it was clearly found that the dry plant α-ketoglutaric-γ-aminobutyric transaminase preparation obtained here catalyses the formation of L-glutamic acid from γ-aminobutyric acid in addition to α-ketoglutaric acid, at pH 7.4, while the same preparation may also promote the conversion of L-glutamic acid to γ-aminobutyric acid and CO_2, at pH 5.8. Concerning the activators, it is demonstrated that both enzyme may contain pyridoxal phosphate.

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[8] J. K. Miettinen and A. I. Vitanen, *Acta. Chem. Scand.*, **7** 1243 (1953) ; *C.A.*, **48**, 13824 (1954).

[9] F. C. Steward, R. G. S. Bidwell and E. W. Yemm, *Nature* **178**, 734 (1956).

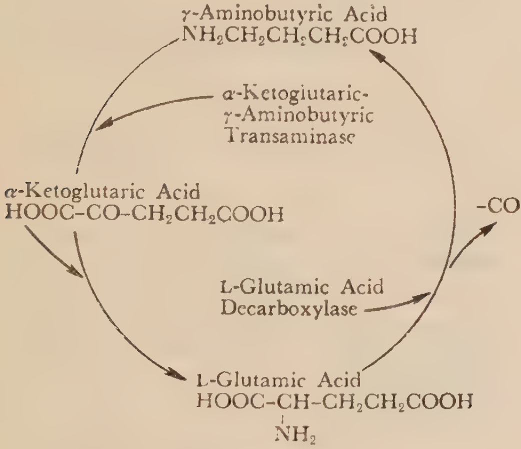
[10] O. Schales and S. S. Schales, *Arch. Biochem. Biophys.*, **69**, 378 (1957).

[11] Y. Sahashi, T. Suzuki, A. Maekawa and T. Miki, *J. Agr. Chem. Soc. Japan*, **29**, 422 (1955).

[12] A. Maekawa and T. Suzuki, *J. Agr. Sci.* (Tokyo Agr. Univ.) **3**, 29 (1956).

Therefore, the results thus obtained seem to suggest the fact that the well known preparation of L-glutamic acid decarboxylase may alternately be convertible to α-ketoglutaric-γ-aminobutyric transaminase preparation, as shown in Scheme 1.

Experiments to determine whether or not both enzymes can be formed by the same kind of protein are now in progress.


γ-Aminobutyric Acid
$NH_2CH_2CH_2CH_2COOH$
α-Ketoglutaric-γ-Aminobutyric Transaminase
α-Ketoglutaric Acid
$HOOC\text{-}CO\text{-}CH_2CH_2COOH$
$-CO_2$
L-Glutamic Acid Decarboxylase
L-Glutamic Acid
$HOOC\text{-}CH(NH_2)\text{-}CH_2CH_2COOH$

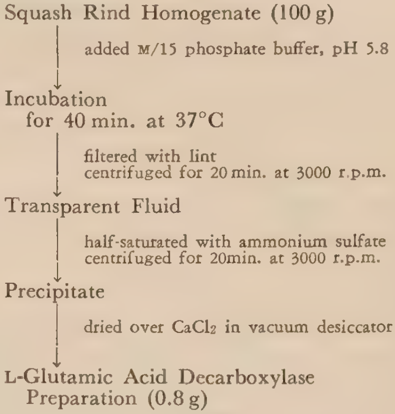
SCHEME 1.

Possible Interconversion of α-Ketoglutaric-γ-aminobutyric Transaminase and L-Glutamic Acid Decarboxylase in Plants.

EXPERIMENTAL

1. Preparation of L-Glutamic Acid Decarboxylase and Estimation of Amino Acids.

Enzyme preparations were first prepared by the method according to Schales and Okunuki[13] (Scheme 2), and the activity of L-glutamic acid decarboxylase in each preparation obtained was tested either by an ordinary Warburg apparatus or by paper partition chromatography. (Fig. 1)


Squash Rind Homogenate (100 g)
added M/15 phosphate buffer, pH 5.8
Incubation for 40 min. at 37°C
filtered with lint
centrifuged for 20 min. at 3000 r.p.m.
Transparent Fluid
half-saturated with ammonium sulfate
centrifuged for 20min. at 3000 r.p.m.
Precipitate
dried over $CaCl_2$ in vacuum desiccator
L-Glutamic Acid Decarboxylase Preparation (0.8 g)

SCHEME 2.

Preparation of L-Glutamic acid decarboxylase Preparation.

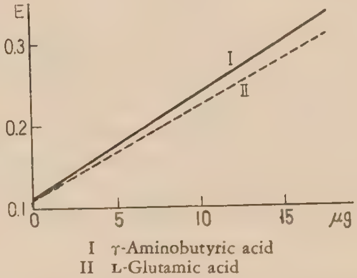


FIG. 1. Relation between the E Values and the Amount of Amino Acids.

Estimation of L-Glutamic and γ-Aminobutyric Acids: The observations were made by application of the ninhydrin color reaction. When the sample is applied for spotting micropipettes ranging in sizes of 0.05 ml to 0.5 ml may be used. When a spray of 1% ninhydrin solution is used to detect the spots, quantities as low as 6 μl of the amino acids are detectable.

The standard experiments were first carried out with 5 μg, 10 μg, 15 μg of standard L-glutamic acid and γ-aminobutyric acid.

Microanalysis was performed by a one-dimensional ascending chromatography, using a strip of Toyo filter paper No. 50 (20 cm in width and 40 cm in length), with acetic acid : *n*-butanol : water (1 : 4 : 2) as the developing solvent. At the end of the experiments, the spots were cut out from paper (2 cm in length at R_F 0.24 for L-glutamic acid and R_F 0.46 for γ-aminobutyric acid), and extracted with 0.5 ml of 0.1 N NaOH.

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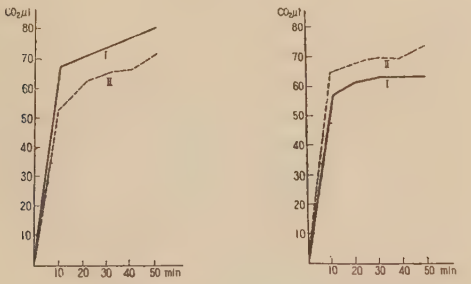
[13] O. Schales, S. Scharles, *Arch. Biochem. Biophys.*, **10**, 455 (1946).

The solution obtained, was adjusted to pH 5.0 with 0.5 ml of citric acid buffer solution, and heated for 20 min. with the addition of $SnCl_2$, ninhydrin solution. Upon cooling, the mixture was diluted with water and acetone and the solution were filled up to 10 ml. The color solution was read in an Erma Type II-A spectrophotometer, filter-57. Standard curves are shown in Fig. 1. The procedure mentioned above was applied in the experiments.

2. Activity Tests of L-Glutamic Acid Decarboxylase Preparation with Addition of Various Factors.

Though it is well known fact that enzyme activity is accelerated by the addition of pyridoxal phosphate, little is yet known concerning other factors. Therefore, several experiments were carried out to investigate the possible effects of vitamin B groups, metalic ions and other compounds.

To the enzyme preparation mentioned above was first added various amounts of vitamin B or the related compounds, and the liberation of CO_2 from pure L-glutamic acid was tested by the Warburg apparatus. As many workers have reported, the addition of pyridoxal phosphate obtained from pyridoxal and ATP always showed an increase of activity, while no positive effects were observed by other B vitamins, pyridoxine, thiamine, nicotinic acid, pantothenic acid and cobalamine, except the inhibitive activity of biotin. (Fig. 2)



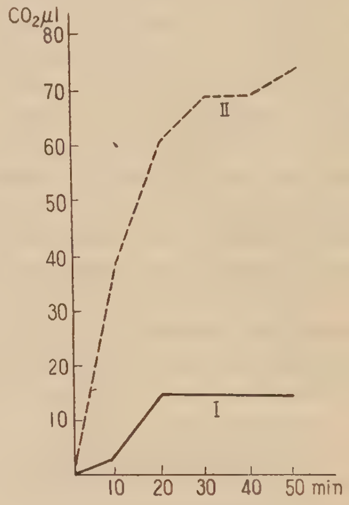
I Pyridoxal+ATP
II Negative control

I Biotin added
II Negative control

FIG. 2. Activity Change of L-Glutamic Acid Decarboxylase by the Addition of either Pyridoxal-Phosphate or Biotin.

In the second experiment, effects of metalic ions were tested, but no significant change was obtained in the CO_2 liberation by the addition of $MgSO_4$, $MnSO_4$, $CoSO_4$ and $CuSO_4$.

In the third experiment, the effects of few organic acids were studied. A marked inhibition was confirmed by the addition of α-ketoglutaric acid, while no effect of maleic acid was observed. (Fig. 3)



I α-KGA added
II Standard

FIG. 3. Inhibition of Liberation of CO_2 by α-Ketoglutaric Acid.

3. Tests on the Reversibility of L-Glutamic Acid Decarboxylase Preparation

It is widely acknowledged that the liberation of CO_2 by L-glutamic acid decarboxylase preparation is almost quantitative. Recently, however, further studies on the reversibility of the enzyme have been repeated under various conditions.

To 1 g of the L-glutamic acid decarboxylase preparation dissolved in 10 ml of M/15 phosphate buffer solution, pH 5.8, was added 1 g of γ-aminobutyric acid, and the mixture was kept for 3 hrs. by saturating with CO_2 gas at pH 5.8. At the end of this experiment, after treating with 5% of metaphosphoric acid, the reaction mixture was centrifuged, and the supernatant thus obtained, was tested for L-glutamic acid formed by paper chromatography. The results are briefly summarized in Table I, showing no reverse action.

Therefore, the action of the L-glutamic acid decarboxylase preparation seems to be constantly irreversible for the quantitative conversion of L-glutamic acid to γ-aminobutyric acid and CO_2.

TABLE I
NEGATIVE TESTS FOR THE REVERSE ACTION OF L-GLUTAMIC ACID DECARBOXYLASE PREPARATION

γ-Aminobutyric acid/10 ml	Source of CO_2/10 ml	Compounds added/10 ml	pH	Time of action	Temp.	Presence of acid Glutamic
1 g	CO_2 gas sat.	—	5.0—8.0 5.8 (mean)	3 hrs.	37°C	—
1	$NaHCO_3$ 100 mg	—	//	//	//	—
1	$KHCO_3$ 100 mg	—	//	//	//	—
1	//	Pyridoxine 10 mg	//	//	//	—
1	//	Biotin 10 mg	//	//	//	—
1	CO_2 60 Atm.	—	//	48 hrs.	//	—
1	//	Biotin 10 mg	//	//	//	—

4. Occurrence of γ-Aminobutyric Acid in Unripe Plant Tissue.

Investigations were extended to the distribution of γ-aminobutyric acid in plant tissue containing L-glutamic acid decarboxylase. Plenty of γ-aminobutyric acid was always detected in unripe plant tissue but only a little amount was found in ripe plant, while no apparent decrease of the decarboxylase activity was observed in the latter. Moreover, it was found that L-glutamic acid decarboxylase occurs in many kinds of edible plants, while, on the contrary, γ-aminobutyric acid is demonstrated in a few species of the vegetable kingdom—Solanaceae such as tomato, nasu, potato. (Fig. 4).

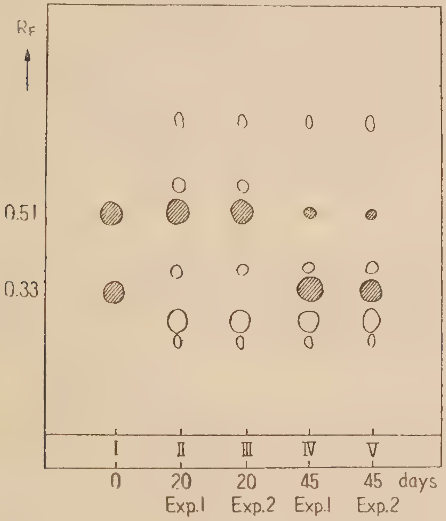


FIG. 4. Decrease of γ-Aminobutyric Acid and Increase of L-Glutamic Acid in Tomato during the Growth Period.

R_F 0.51—γ-Aminobutyric acid
R_F 0.33—L-Glutamic acid
Acetic acid : *n*-Butanol : Water (1 : 4 : 2)

I Standard; II, III Unripe tomato;
IV, V Ripe tomato.

5. Occurrence of L-Glutamic Acid in Edible Plant Tissue.

Our co-workers carried out several studies on the distribution of L-glutamic acid in the plants containing L-glutamic acid decarboxylase. After cautious experiments, it was confirmed that L-glutamic acid is always detectable in almost all kinds of edible plants in which L-glutamic acid decarboxylase appears. Especially, the amount of L-glutamic acid was found to be maximum prior to harvesting. (Fig. 4)

6. Recovery of L-Glutamic Acid Decarboxylase System inhibited by α-Ketoglutaric Acid.

A quantity of 20 mg of L-glutamic acid and 80 mg of α-ketoglutaric acid was first dissolved in 100 ml of water, and the mixture was used for a test. To 35 ml of the sample solution described above was added 1 ml of conc. HCl and it was subsequently evaporated under diminished pressure to dryness at 40°C. The residual syrup was again dissolved in 2 ml of 5% HCl, and completely extracted 5 times with 4 ml of ether, each time. The aqueous layer was evaporated in vacuo, and to the residual mass was poured 10 ml of water. The solution thus obtained, may be available for the determination of L-glutamic acid by L-glutamic acid decarboxylase according to the Warburg method. The result is as shown in Fig. 5. Plant L-glutamic acid decarboxylase was confirmed to catalyse the liberation of CO_2 from L-glutamic acid almost quantitatively in the case where the former is free from α-ketoglutaric acid.

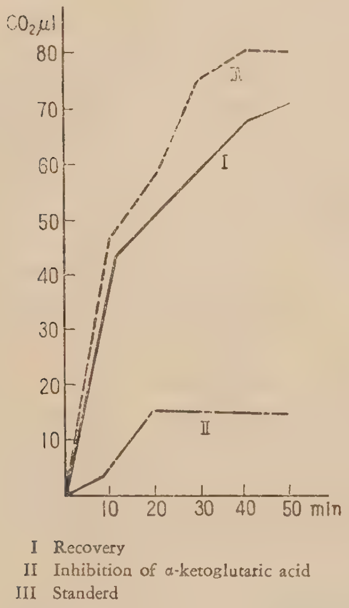


FIG. 5. Recovery of the L-Glutamic Acid Decarboxylase System inhibited by α-Ketoglutaric Acid.

7. Tests on the Reverse Action of L-Glutamic Acid Decarboxylase with freshly prepared Supernatant Fluid of Plant Tissue.

Experiments were repeated with freshly prepared supernatant fluid of plant tissue containing L-glutamic acid decarboxylase. 50 g of potato was pulverized in 50 ml of M/15 phosphate buffer, pH 5.0 and 7.0, and the homogenate was centrifuged for 30 min., at 3000 r.p.m.. To 20 ml of the supernatant fluid thus obtained, was added 1 g of γ-aminobutyric acid, and the mixture was kept for 3 hrs. by constant saturation of CO_2 gas at 37°C. After the reaction was completed, the solution was treated with 5% metaphosphoric acid, then the transparent upperlayer was tested for the formation of L-glutamic acid. (Table II)

As it is seen from the data shown in Table II, the reverse action was markedly recognized in each experiment in the presence of trace impurities. Consequently, the phenomenon seems to suggest a fact of especial interest that there may possiblly be another metabolic pathway besides the single L-glutamic acid decarboxylase system.

8. Occurrence of α-Ketoglutaric-γ-Aminobutyric Transaminase in Edible Plant Tissue containing L-Glutamic Acid Decarboxylase.

During these investigations, it has been suggested that there may also be an enzyme α-ketoglutaric-γ-aminobutyric transaminase in the same plant tissue containing L-glutamic acid decarboxylase. A α-ketoglutaric-γ-aminobutyric transaminase preparation was first prepared according to ordinary method, at pH 7.4.

To 50 g of potato homogenate was added 50 ml of M/15 phosphate buffer, pH 7.4, and the mixture was kept at 37°C for 2 hrs.. After it was filtered with lint, the supernatant layer was centrifuged for 10 min. at 3500–4000 r.p.m.. To 5 ml of the trans-

TABLE II

FORMATION OF L-GLUTAMIC ACID FROM γ-AMINOBUTYRIC ACID BY FRESH SUPERNANT FLUID OF PLANT TISSUE CONTAINING L-GLUTAMIC ACID DECARBOXYLASE UNDER VARIOUS CONDITIONS

Treatments				Appearance of L-Glutamic Acid
CO_2 gas saturated	Temp.	Time	Co-factors added	
−	37°C	3 hrs.	none	+
+	37	24	〃	+
−	37	24	〃	+
+	0	24	〃	+
−	0	24	〃	+
+	Room temp.	24	〃	+
−	do	24	〃	+
+	37	24	Pyridoxine 10 mg/20 ml of the enzyme prep. added	+
−	37	24	〃	+
+	0	24	〃	+
−	0	24	〃	+

TABLE III
FORMATION OF L-GLUTAMIC ACID FROM γ-AMINOBUTYRIC ACID WITH α-KETOGLUTARIC ACID BY TRANSAMINATION OF PLANT FLUID CONTAINING L-GLUTAMIC ACID DECARBOXYLASE

Kinds of Plants	Total L-Glutamic acid in the Reaction Mixture	L-Glutamic acid in the Enzyme Preparation	L-Glutamic acid formed	L-GA/γ-ABA
Potato Fluid	8913 μg	2249 μg	6664 μg	6.66%
	7914	2083	5831	5.83

parent enzyme solution thus obtained, was added 50 mg α-ketoglutaric acid and 100 mg γ-aminobutyric acid/5 ml of M/15 phosphate buffer solution, and on incubation for 24 hrs. at 37°C, the L-glutamic acid formed was determined by paper chromatography, using Toyo filter paper No. 50, acetic acid : *n*-butanol : water (1 : 4 : 2), and by ninhydrin coloration photometry, using an Erma Type II-A photometer, filter-57. (Table III)

9. Effects of pH, Temperature and Co-factors on the Potato Enzyme Fluid.

Effects of pH on the formation of L-glutamic acid in the enzyme solution was studied. Maximum formation was observed at pH 7.4~8.4, and at a rather low pH 6.6. (Table IV)

TABLE IV
EFFECT OF pH ON THE FORMATION OF L-GLUTAMIC ACID BY THE POTATO ENZYME FLUID

pH	L-Glutamic acid formed in 1 ml	L-GA/γ-ABA
6.6	919 μg	2.51%
7.4	1286	4.35
8.4	1286	4.35

In separate experiments concerning the temperature and co-factors, it was found that a low temperature is optimum (Table V), and that pyridoxal phosphate may be markedly active in producing an excellent yield. (Fig. 6)

TABLE V
EFFECT OF TEMPERATURE ON THE FORMATION OF L-GLUTAMIC ACID BY FLUID POTATO ENZYME

Temperature	L-Glutamic Acid formed in 1 ml	L-GA/γ-ABA
0°C	1035 μg	3.09%
37	918	2.51
Room temp.	835	2.09

10. Isolation of α-Ketoglutaric-γ-Aminobutyric Transaminase Preparation.

Fresh transparent fluid was first prepared from Squash-Rind homogenate as shown in Scheme 2, and after adjusting the fluid to pH 5.8–8.4 with M/15 phosphate buffer, this transaminase preparation was isolated by the following process. (Scheme 3)

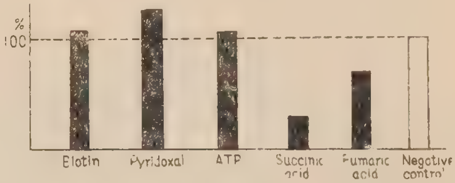


FIG. 6. Effect of Pyridoxal on the Formation of L-Glutamic Acid by Potato Fluid.

Squash Rind Homogenate
↓ added M/15 Phosphate buffer, pH 7.4
Incubation for 40 min. at 37°C
↓ filtered with lint centrifuged for 20min. at 3000 r.p.m.
Transparent Fluid
↓ half-saturated with ammonium sulfate centrifuged for 20min. at 3000 r.p.m.
Precipitate
↓ dried over $CaCl_2$ in vaccum desiccator
α-Ketoglutaric-γ-Aminobutyric Transaminase Preparation

SCHEME 3.
Preparation of α-Ketoglutaric-γ-Aminobutyric Transaminase Preparacion.

A portion of 0.5 g of the α-ketoglutaric-γ-aminobutyric transaminase preparation was dissolved in 10 ml of M/15 phosphate buffer solution of pH 7.4, to which 103 mg of γ-aminobutyric acid and 73 mg of α-ketoglutaric acid were added; after incubation for 24 hrs. at 37°C, the L-glutamic acid formed was estimated as mentioned above by paper chromatography, using Toyo filter paper No. 50, acetic acid : *n*-butanol : water (1 : 4 : 2), and by ninhydrin coloration photometrically, using an Erma Type II-A photometer, filter-57. (Table VI)

TABLE VI

FORMATION OF L-GLUTAMIC ACID FROM γ-AMINOBUTYRIC ACID WITH α-KETOGLUTARIC ACID BY PLANT α-KETOGLUTARIC-γ-AMINOBUTYRIC TRANSAMINASE PREPARATION OBTAINED FROM SQUASH-RIND FLUID

Kinds of Plants	Total L-glutamic acid in the Reaction Mixture	L-Glutamic acid in the Enzyme Preparation	L-Glutamic acid formed	L-GA/γ-ABA
Squash Rind	5320 μg	0 μg	5320 μg	5.16%
	8750	0	8750	8.47

SUMMARY

(1) From our view point, several investigations of the reverse action of L-glutamic acid decarboxylase occuring in plants were attempted, and the distribution and activity of L-glutamic acid decarboxylase in 50 kinds of edible plants were tested. During these experiments, severe inhibition of α-ketoglutaric acid was recognized, besides activation of the enzyme action by pyridoxal phosphate, and the occurrence of L-glutamic acid and γ-aminobutyric acid was always found in the same plant tissue.

(2) From repeated experiments, it has been made clear that α-ketoglutaric-γ-aminobutyric transaminase may be demonstrated in the same plant tissue containing L-glutamic acid decarboxylyase, and finally we succeeded to confirm α-ketoglutaric-γ-aminobutyric transaminase in the L-glutamic acid decarboxylase as it is reported in many papers.

(3) Moreover, it was confirmed that the dry plant α-ketoglutaric-γ-aminobutyric transaminase preparation obtained here catalyses the biosynthesis in vitro of L-glutamic acid from γ-aminobutyric acid in addition of α-ketoglutaric acid, at pH 7.4, while the same preparation may also activate the conversion of L-glutamic acid to γ-aminobutyric acid and CO_2, at pH 5.8.

Acknowledgements. The expense of this research was partly defrayed from a grant in aid for Fundamental Scientific Research of Ministry of Education and the Ajinomoto Co. Inc., to which the authors' thanks are due. The authors also wish to thank Messrs T. Miki, T. Nakae, S. Ogata, T. Nomoto and T. Hiroishi of this laboratory for their assistance in carrying out the experiments.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 1, p. 47~53, 1957]

Some Volatile Acids and Esters of Cigarette Smoke*

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Received October 8, 1957

The isolation and identification of volatile organic acids and esters contained in the cigarette smoke, which was produced in constant volume continuous smoking by an artificial smoking device, have been studied. Acetic, propionic, *n*-butyric, isovaleric and β-methyl valeric acid as their *p*-bromophenacyl esters, and benzoic acid as the crystal of itself, were isolated and identified by mixed examinations, paper chromatographic techniques, infrared spectra and elementary analyses; and the presence of *n*-caproic acid was estimated. By paper chromatography, ethyl esters of acetic and propionic acid were qualitatively identified, and ethyl esters of *n*-butyric, isovaleric, and *n*-caproic and/or β-methylvaleric acid were estimated. Among these compounds, β-methylvaleric acid and all of the esters have not yet been reported in the literature concerning tobacco smoke.

In a previous paper1) it has already been reported that acidic fractions (I) and (II) were obtained by the fractionation of smoke-components which were produced in constant volume continuous smoking of cigarettes by an artificial smoking device (22,000 regular-size uncased cigarettes; blend, 72.0–23.1–4.9 blend of Japanese and Indian Flue-cured, Japanese Native, and Indian Native tobacco leaves; weight ranges of filled cut-leaves, 1.078 g±5%; moisture content, 10.0%; smoking volume, 20 cc/sec./1 cigt.; length of butts, 23 mm).

The present paper deals with the isolation, identification and estimation of the individual volatile organic acids and esters contained in these two fractions.

EXPERIMENTAL

A portion of the acidic fraction (I) (23.7 g, equivalent to the acidic fraction of the smoke-components of 10,475 cigarettes) was steam-distilled to divide it into a steam-distillable part (23.20 g) and the residue. Similarly, the steam-distillation of the acidic fraction (II) (21.20 g, corresponding to the smoke of 10,475 cigarettes) gave 11.08 g of a steam-distillable part.

I. Volatile Acids

1. Acidic Fraction (I): The steam-distillable part of the acidic fraction (I) (23.20 g) was fractionally distilled under ordinary pressure, and fractions Fr. 1, Fr. 2 and Fr. 3 were obtained (Table 1).

TABLE I

FRACTIONAL DISTILLATION OF THE STEAM-DISTILLABLE PART OF ACIDIC FRACTION (I), AND THE ACID CONTENTS OF EACH FRACTION

	No. of Fraction	B.p. (°C/760 mm Hg)	Amounts of Fractions (g)	Acid Content* (% as acetic acid)
	Fr. 1	41– 90	6.25	0
	Fr. 2	90–120	14.50	69.7
Redistn.	Fr. 2–1	65–100	2.4	17.9
	Fr. 2–2	100–105	1.5	47.9
	Fr. 2–3	105–107	5.6	74.8
	Fr. 2–4	107–120	1.9	75.3
	Fr. 2–5	120–	0.9	—**
	Fr. 3	120–	2.30	—**

* Titration with N/10-NaOH; ** Impossible to titrate owing to the dark color.

For the preliminary investigations of volatile acids in each fraction, diethylamine salts of acids were paper-chromatographed according to A. R. Jones et al.2) [Filter paper, Whatman No. 1; solvent, *n*-butanol : water : diethylamine (100 : 15 : 1 by vol.); spraying reagent, citric acid-acidic alcohol solution of 0.05%

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* Part VI of a series of papers entitled "Studies on Tobacco Smoke".

1) Masao Izawa and Yûsuke Kobashi, This Bulletin, **21**, 357 (1957).

2) A. R. Jones, E. J. Dowling and W. J. Skraba, *Anal. Chem.*, **25**, 394 (1953).

bromocresol green (pH 5.5)], and spots that appeared on the chromatogram were compared with the spots of authentic formic, acetic, propionic, *n*-butyric, isobutyric, *n*-valeric and isovaleric acid.

Because the fraction Fr. 1 gave almost no spots on the chromatogram and its titration value with N/10-sodium hydroxide was practically zero, the presence of aromatic ester-like substances other than acids was suspected. The greater part of the fraction Fr. 2, having an acid content of 69.7% as acetic acid, consisted of acetic or formic acid (R_F values of both acids were almost equal). In the fraction Fr. 3, the spots corresponding to formic, acetic and propionic acid were not observed, but *n*- or isobutyric, *n*- or isovaleric and a higher boiling acid were estimated (R_F values of *n*-acid and isoacid were almost equal). The measurement of acid contents, however, was impossible owing to the dark brown coloration of this fraction.

The fraction Fr. 2 was furthermore fractionally redistilled into five fractions (Table I), which were paper-chromatographed into a similar manner described above[2], respectively (Fig. 1). Both fractions Fr. 2-1 and Fr. 2-2, showing the presence of acetic or formic acid and propionic acid on the chromatogram, were set aside for investigations of esters because of their lower acid contents. Also, in fractions Fr. 2-3 and Fr. 2-4, the spots equivalent to butyric and valeric acid were observed besides those of acetic or formic acid. Concerning fraction Fr. 2-5, a small spot, showing the presence of another higher-boiling acid in addition to the acids contained in the other fractions, was observed.

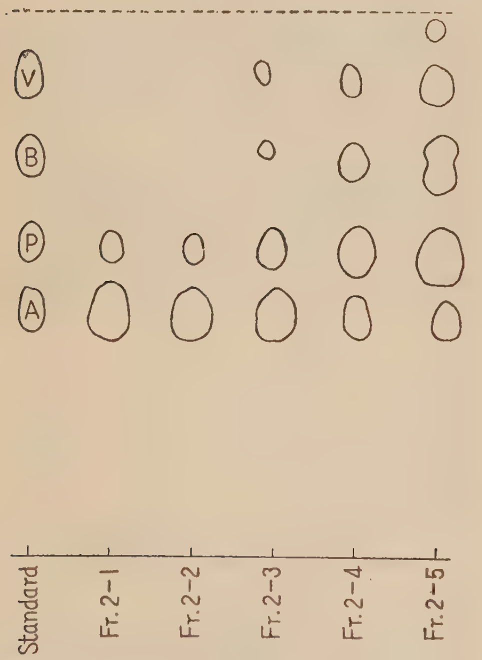

FIG. 1. Paper Chromatography of Each Fraction of Fr. 2.

Filter paper, Whatman No. 1; Solvent, *n*-butanol : water : diethylamine (100 : 15 : 1 by vol.); Spraying reagent, B.C.G.; Standard, A— acetic and formic acid, P— propionic acid., B— *n*- and *iso*-butyric acid, V— *n*- and *iso*-valeric acid.

Isolation and Identification of Acetic Acid: As the fractions Fr. 2-3 and Fr. 2-4 contained the same acids, the former fraction possessing a comparatively higher acid percentage and larger amounts, was liquid-chromatographed following the procedure given by F. A. Vandenheuvel et al.[3] for separating individual acids. After 26 g of silicic acid (Mallinckrodt, analytical reagent, No. 2847, specially prepared for chromatography, 100-mesh) was thoroughly mixed with 16 ml af an alkaline methanolic solution containing 7.5 ml of 1N-sodium hydroxide in 1 liter, the slurry was poured into a chromato-column (17×400 mm), using about 70 ml of isooctane. To settle the slurry in the column, an excess solvent was removed by applying a nitrogen gas pressure, and a silicic acid-packed column of about 200 mm in length was obtained. The fraction Fr. 2-3 (253 mg) was placed on the top of the column, developed with isooctane at flow-rate of 2-3 ml per min. under a nitrogen gas pressure of about 250 mm Hg. A 10% solution of ether in isooctane and absolute ether were also used as developing solvents, successively. A fraction collector being employed, each 5 ml of eluates was collected into test tubes, followed by titration with 2N/100-sodium hydroxide using phenolphthalein or phenol red (Fig. 2).

From eluate B, *p*-bromophenacyl esters of volatile acids were prepared in the usual manner[4]. A crystal of m.p. 85-86°C was obtained after recrystallizations with ethanol. The elementary analyses of this crystal showed the following values and its m.p. did not change by admixture with *p*-bromophenacyl ester of an authentic acetic acid (m.p. 85.5-86.5°C). The infrared spectra and elementary analyses of the two compounds were identical.

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[3] F. A. Vandenheuvel and E. R. Hayes, *Anal. Chem.*, **24**, 960 (1952).

[4] R. L. Shriner, R. C. Fuson and D. Y. Curtin, "The Systematic Identification of Organic Compounds", 4th Ed., John Wiley & Sons, Inc., 1956, p. 200.

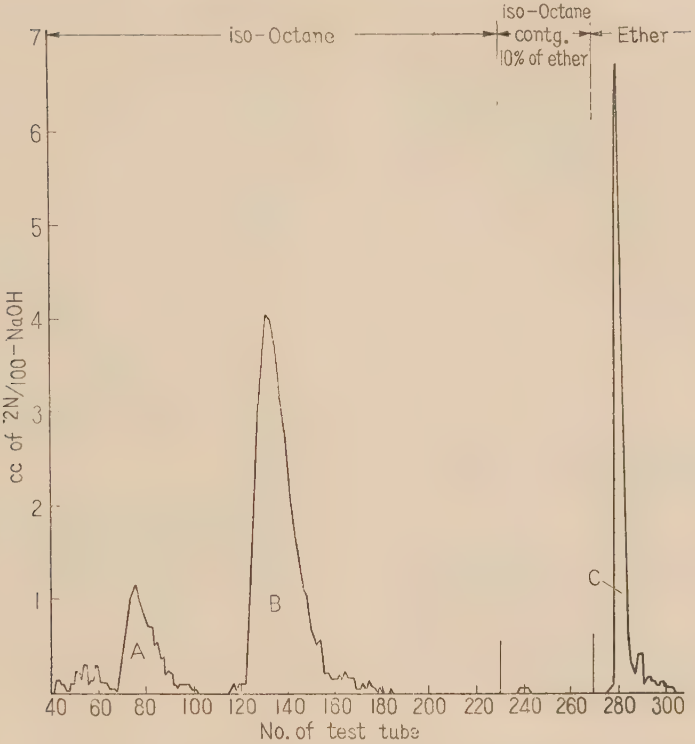

FIG. 2. Liquid-Chromatography of Fraction Fr. 2–3.

Found: C, 47.12; H, 3.81

Calcd. for $C_{10}H_9O_3Br$: C, 46.72; H, 3.52

In the same way, acetic acid was identified in eluate C by mixed examinations and infrared spectra.

The acids contained in eluate A were not identified owing to their small quantities.

2. Acidic Fraction (II): For the reason that paper chromatography[2)] of diethylamine salts of acids indicated that the steam-distillable part of the acidic fraction (II) possesses acids exactly similar to those contained in the fractions Fr. 2–5 and Fr. 3 shown in Table I, these three fractions were combined and fractionally distilled (Table II). Acids in each fraction were preliminary investigated by paper chromatography of their diethylamine salts (Fig. 3). It was estimated that fraction Fr. M–1 did not contain any acids; Fr. M–2, Fr. M–3 and Fr. M–4 each contained acetic (and/or formic) and propionic acid; Fr. M–5 comprised propionic acid and small amounts of *n*- or iso-butyric acid; and Fr. M–6 or above fractions contained higher-boiling acids.

TABLE II

FRACTIONAL DISTILLATION OF MIXTURE OF THE STEAM-DISTILLABLE PART OF ACIDIC FRACTION (II); FRACTION FR. 2–5 AND FRACTION FR. 3 OF ACIDIC FRACTION (I)

No. of Fraction	B.p. (˚C/mm Hg)	Amounts of Fractions (g)	Acid Content* (%)
Fr. M–1	59– 86/760	1.88	0.0
Fr. M–2	86–110/760	0.47	16.3 (A)
Fr. M–3	110–130/760	0.48	77.9 (P)
Fr. M–4	130–145/760	1.72	102.5 (P)
Fr. M–5	60– 80/30	0.88	85.0 (P)
Fr. M–6	80– 92/30	0.62	85.9 (B)
Fr. M–7	92–100/30	0.43	101.4 (V)
Fr. M–8	100–110/30	0.47	90.2 (V)
Fr. M–9	110–115/30	0.64	99.5 (C)
Fr. M–10	115–155/30	0.72	78.7 (C)
Fr. M–11	——**	0.46	—
Fr. M–12	Residue	1.51	—

* Titrated with N/10-NaOH and calculated for acetic (A), propionic (P), *n*-butyric (B), *n*-valeric (V) and caproic acid (C), respectively; ** Crystallized out in the cooler.

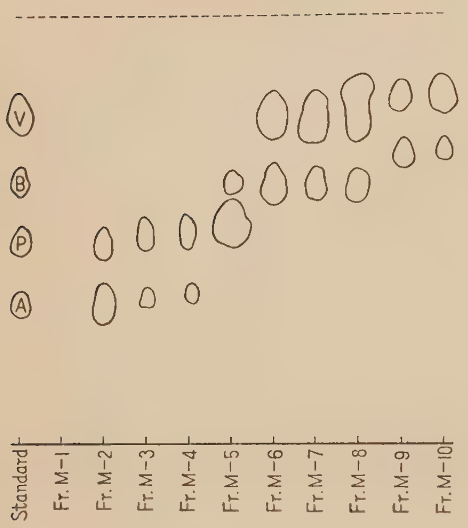

FIG. 3. Paper Chromatography of Fractional Distillates of Mixture of the Steam-distillable Part of Acidic Fraction (II), Fraction Fr. 2–5 and Fr. 3.

Filter paper, Whatman No. 1; Solvent, *n*-butanol: water: : diethylamine (100:15:1 by vol.); Spraying reagent, B.C.G.; Standard, A—Acetic and formic acid, P—propionic acid, B—*n*- and isobutyric acid, V—*n*- and isovaleric acid.

Isolation and Identification of Propionic Acid: Because the spot equivalent to propionic acid in the fraction Fr. M–5 was found to be the largest among all of the fractions described above, Fr. M–5 was employed to identify this acid. Crude *p*-bromophenacyl esters from Fr. M–5 were repeatedly recrystallized with ethanol until the constant m.p. of 58°C was attained. The elementry analysis values of this crystal are shown below. The m.p. of this crystal did not depress by admixture with *p*-bromophenacyl ester of an authentic propionic acid (m.p. 59°C), and the infrared spectra and elementary analyses of the two derivatives were identical.

Found; C, 49.38; H, 4.04

Calcd. for $C_{11}H_{11}O_3Br$: C, 48.73; H, 4.09

Isolation and Identification of Isovaleric Acid: A mixture of the fractions Fr. M–6 and Fr. M–7 (273 mg) was liquid-chromatographed3), and each 5-ml of eluates was titrated with 2N/100-sodium hydroxide (Fig. 4). After recrystallizations of *p*-bromophenacyl esters obtained from eluate A with ethanol, a crystal of m.p. 67°C was gained. The elementary analysis values of this crystal are given below. The m.p., infrared spectrum and elementary analysis values of this crystal agreed closely with those of *p*-bromophenacyl ester of an authentic isovaleric acid (m.p. 67–68°C).

Found: C, 52.52; H, 4.95

Calcd. for $C_{13}H_{15}O_3Br$: C, 52.19; H, 5.05

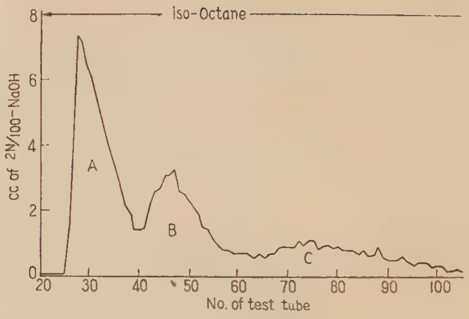

FIG. 4. Liquid-Chromatography of Mixture of Fractions Fr. M–6 and Fr. M–7.

Isolation and Identification of *n*-Butyric Acid: A crystal of *p*-bromophenacyl easter of m.p. 63°C was obtained after repeated recrystallizations of crude *p*-bromophenacyl esters prepared from eluate B. No depression of m.p. was observed by admixture of any pair of this crystal and *p*-bromophenacyl ester of an authentic *n*-butyric acid (m.p. 63°C). Elementary analyses could not be carried out owing to the very small amounts of the sample. However, the infrared spectrum, and its spot position on the paper-chromatogram agreed with the spot of *p*-bromophenacyl ester of an authentic *n*-butyric acid. These evidences proved the presence of *n*-butyric acid.

The acids in eluate C were not identified owing to the very small quantities of their *p*-bromophenacyl esters after recrystallizations.

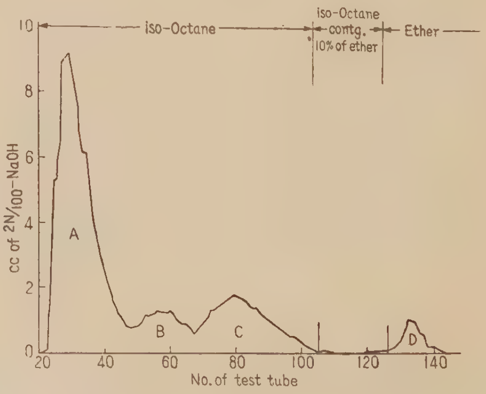

FIG. 5. Liquid-Chromatography of Fraction Fr. M–8.

Isolation and Identification of β-Methylvaleric Acid: The fraction Fr. M-8 (470 mg) was liquid-chromatographed[3] (Fig. 5). After repeated recrystallizations with 60% ethanol, a *p*-bromophenacyl ester having m.p. 38–39.5°C was obtained from eluate A. (Recrystallizations with absolute ethanol gave a crystal of m.p. 42.5–43.5°C). The elementary analysis value of this crystal (m.p. 38–39.5°C) are given below, and the m.p. of this crystal was not depressed by admixture with *p*-bromopeenacyl ester of an authentic β-methylvaleric acid (m.p. 39–40°C). The elementary analyses and infrared spectra of the two specimens were also found to be identical.

Found: C, 52.75; H, 5.37

Calcd. for $C_{14}H_{17}O_3Br$: C, 53.69; H, 5.47

Acids in eluates B, C and D have not yet been investigated.

Isolation and Estimation of *n*-Caproic Acid: It was found that the fraction Fr. M-9 and Fr. M-10 contained the same acids. Then, 425 mg of Fr. M-10 was liquid-chromatographed[3] (Fig. 6). Eluate A, from which *p*-bromophenacyl esters were prepared and repeatedly recrystallized with ethanol, gave a crystal of m.p. 48–50°C. In spite of repeated recrystallizations, the m.p. of the crystal did not agree with the m.p. (72°C) of *p*-bromophenacyl ester of an authentic *n*-caproic acid described in the literature; but spot position on paper chromatograms, infrared spectra and elementary analyses of the both specimens closely agreed with each other.

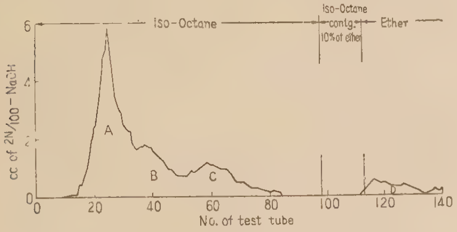

FIG. 6. Liquid-Chromatography of Fraction Fr. M-10

Found: C, 54.07; H, 5.43

Calcd. for $C_{14}H_{17}O_3Br$: C, 53.69; H, 5.47

From these evidences it was estimated that this crystal is a *p*-bromophenacyl ester of *n*-caproic acid, in which persistent impurities, perhaps *p*-bromophenacyl esters of isomers of *n*-caproic acid, were included. No analyses have yet been carried out on eluates B, C and D.

Isolation and Identification of Benzoic Acid: The fraction Fr. M-11 (460 mg), which had crystallized out in the cooler in the fractional distillation, was recrystallized from water and a crystal of m.p. 118–119.5°C was obtained. The values of elementary analyses of this crystal are given below and no change of m.p. of this crystal was observed by admixture with an authentic benzoic acid purified by sublimation (m.p. 118–119°C). The infrared spectra and elementary analyses of these two specimens were in close agreement with each other.

Found: C, 68.86; H, 4.94

Calcd. for $C_7H_6O_2$: C, 68.84; H, 4.95

Analyses on the residue (Fr. M-12) were not attempted owing to its dark coloration and sticky properties.

II. Esters

Because some fractions shown in Tables I and II have fragrant and ester-like odors and their acid contents as acetic acid were comparatively small, these fractions were paper-chromatographed according to the Inoue and Noda hydroxamic acid procedure for esters[5]. To about 50–100 mg of each sample, a mixture of 0.6 ml of a 5% hydroxylamine hydrochloride-ethanolic solution and 0.5 ml of a 10% potassium hydroxide-ethanolic solution were added, and the mixture was heated at 80–85°C under reflux for 10 min.

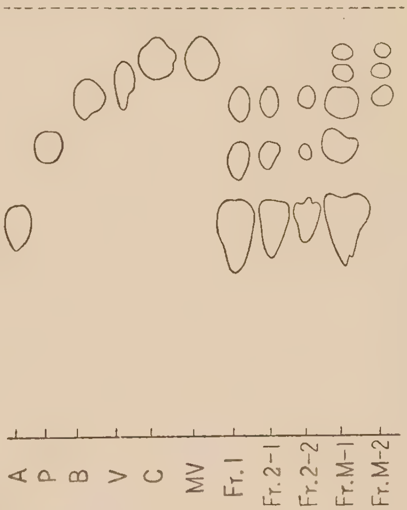

FIG. 7. Paper Chromatography of Esters in Low Acid Content-Fractions According to the Inoue and Noda Hydroxamic Acid Procedure for Esters[5].

Standard: A— Ethyl acetate, P— Ethyl propionate, B— Ethyl *n*-butyrate, V— Ethyl *iso*-valerate, C— Ethyl *n*-capronate, MV— Ethyl β-methylvalerate

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5) Y. Inoue and M. Noda, *J. Agr. Chem. Soc. Japan*, **24**, 291 (1951).

After the reaction mixture was filtrated, the precipitates on a filter paper were washed with small amounts of ethanol. The filtrates and washings were combined, evaporated to almost dryness, slightly acidified by dropping an ethanol-hydrochloric acid (2:1) solution and the supernatant was paper-chromatographed with water-saturated *n*-butanol by an ascending technique (Fig. 7). The paper chromatogram indicated that esters of acetic, propionic and butyric acid were present in Fr. 1, Fr. 2–1 and Fr. 2–2; esters of acetic, propionic, buyric, valeric and caproic acid in Fr. M–1; and esters of butyric, valeric and caproic acid in Fr. M–2. Concerning the butyric, valeric and caproic acid, the discrimination of these *n*-acids from their isomers was impossible in the above-described paper chromatography. Therefore, a greater portion of each fraction was dissolved into ether, removed admixed free acids with a sodium bicarbonate solution, saponified with 25% sodium hydroxide solution by heating, and other paper chromatographies, employing diethylamine salts[2)] or hydrazides[6)] of these acids, were carried out. But these chromatographies were also unsuccessful in the discrimination of *n*-acids from their isomers owing to their identical R_F values. As described in the preceding paragraphs, however, *n*-butyric, isovaleric, *n*-caproic and β-methylvaleric acid had been identified or estimated, so these acids are estimated to be *n*-butyric, isovaleric, *n*-caproic and/or β-methylvaleric acid.

On the other hand, to investigate alcohol components of the esters, the alcohol components obtained by the saponification were extracted with ether by shaking, the ethereal layer was evaporated, and the evaporation residue was paper-chromatographed according to the manner of T. Kariyone et al.[7)]. The sample solution was treated with ether containing 0.1–0.4 cc of carbon disulfide in the rate of 20% and about 0.1 g of potassium hydroxide, then, shaked for 5–15 min.. After the supernatant was transferred into a test tube and distilled off the solvent, the residue was dissolved into 0.01–0.05 cc of water and paper-chromatographed employing a mixture of *n*-butanol: 2% potassium hydroxide (1:1) as the solvent by an ascending technique. After development, the paper was dried and observed under ultra violet light irradiation. Because every fraction gave only a single spot equivalent to ethyl alcohol (R_F value, 0.27) on the paper chromatogram, the alcohol component of these esters was found to be ethyl alcohol.

From these experiments ethyl acetate and ethyl propionate were qualitatively identified, and ethyl esters of *n*-butyric, isovaleric, *n*-caproic and/or β-methylvaleric acid were estimated.

DISCUSSION

Several authors have already published reports on organic acids found in tobacco smoke[8,9,10,11)]. Yu. O. Gabel et al.[8)] found formic, acetic, propionic acid and an unsaturated acid $C_{10}H_{12}O_2$ in the drydistilled tobacco smoke. C. Neuberg et al.[9)] confirmed the existence of formic, acetic, butyric, valeric, caproic, C_7- and C_8-acid in the smoke of pipe tobacco. J. A. Bradford et al.[10)] discovered formic and acetic acid in the cigarette smoke produced by mechanical smoking. Recently, D. A. Buyske et al.[11)] studied on the organic acids in the smoke of cigarttes made of Flue-cured, Burley or Turkish leaf tobaccos. They chromatographically separated each acid from the smoke produced by mechanical smoking and quantitatively determined formic, acetic, propionic, butyric or isobutyric, valeric or isovaleric, caproic, heptylic, caprylic, nonylic or capric, higher C- and benzoic acid.

Excluding the study of C. Neuberg et al., these authors, however, did not carry out identification through the derivatives of the acids. The present authors have intended to isolate and identify volatile organic acids from the acid fractions of cigarette smoke as their derivatives or the crystal itself. By conducting these experiments acetic, propionic, *n*-butyric, isovaleric and β-methyl-valeric, and benzoic acid were isolated and identified, and the existence of *n*-caproic acid were estimated.

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6) K. Satake and T. Seki, *Kagaku no Ryôiki* (*J. Japan. Chem.*), **4**, 557 (1950).

7) T. Kariyone, Y. Hashimoto and M. Kimura, *J. Pharm. Soc. Japan*, **73**, 1093 (1953).

8) Yu. O. Gabel and G.I. Kipriyanov, *Ukrainskii Khem. Zhur.*, *4*, *Tech. Pt.*, 45 (1929); *Chem. Abstr.*, **24**, 916 (1930).

9) C. Neuberg und J. Burkard, *Biochem. Z.*, **243**, 472 (1931).

10) J. A. Bradford, E. S. Harlow, W. R. Harlan and H. R. Hanmer, *Ind. Eng. Chem.*, **29**, 45 (1937).

11) D. A. Buyske, P. Wilder, Jr., and M. E. Hobbs, *Anal. Chem.*, **29**, 105 (1957).

Formic acid, which was found by all of the above authors but was not isolated in the present experiment, is to be studied in a more exact and detailed analysis. Higher C-acids, which were not isolated as their derivatives probably due to their very small amounts, must be investigated in the future.

The existence of substances having ester-like odor are estimated from the aroma of cigarette smoke itself, but nobody has yet confirmed the esters in the smoke. In this experiment ethyl esters of acetic and propionic acid were qualitatively identified and ethyl esters of *n*-butyric, isovaleric, and *n*-caproic and/or β-methylvaleric acid were estimated. These esters play an important role in the manifestation of sweet aroma and complicating the aroma of cigarette smoke. Also, the volatile organic acids, which exist in comparatively larger amounts, are responsible for the good flavor of cigarette smoke.

Acknowledgement. The authors wish to express their sincere thanks to Prof. Y. Obata of Hokkaido University for his constant guidance and encouragement during the course of this work, and to Director T. Nakajima and Chief I. Ônishi of this Research Institute for their encouragement and support, and also to Dr. T. Yabuta, Emeritus Prof. of University of Tokyo, for his interest and valuable advice. Indebts are also due to the Laboratory of Microanalysis, Department of Agricultural Chemistry, University of Tokyo, for the microanalyses, and to Miss. M. Motomatsu and Mrs. H. Hôshaku for their assistance in carrying out the experiments.

Note

Effect of Gibberellins on Ehrlich Mouse Carcinoma

(Biochemistry of "Bakanae" Fungus. Part 43)

Although gibberellins show a very characteristic physiological activity on plant tissues, no activity has been observed on microorganisms[1] and chick embryo[2,3], so far as our experiments up to the present are concerned. With the hope that there might be some effects of gibberellins exceptionally on neoplastic cells, the following experiment was carried out but the results were not fruitful.

One million of Ehrlich mouse carcinoma cells was inoculated intraperitoneally to each of mice. After two hours a group of 8 mice was intraperitoneally injected with 180 mcg gibberellin A/mouse/day, another group with 18 mcg gibberellin A/mouse/day and the control group with physiological saline for a period of 8 days. The results obtained are shown in the following figure. By the method employed, any inhibition of ascites increase and prolongation of the survival time of animals by gibberellin A were not observed.

Gibberellin A, used in this experiment, melts at 229–32°C (dec.) and is a mixture of gibberellins A_1 66.6%, A_2 0.0%, A_3 17.9% and A_4 12.9%. Gibberellin A_4 is one of gibberellins, newly discovered from the broth filtrate of tank culture of *Gibberella fujikuroi*, and its physical and chemical properties as well as its physiological activities will be soon published.

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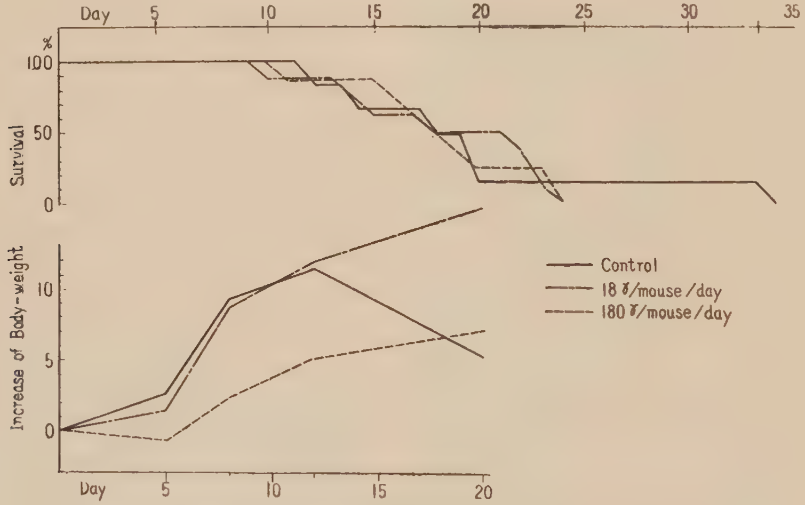

FIG. Effect of Gibberellin A on Ascitic Form of Ehrlich Mouse Carcinoma

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[1] T. Hayashi, *Jour. Agr. Chem. Soc. Japan*, **16**, 386 (1940).

[2] A. Kawarada et al, *Bull. Agr. Chem. Soc. Japan*, **19**, 278 (1955).

[3] T. Yabuta et al, *ibid.*, **18**, 208 (1942).

On the MG Values in Paper-electrophoresis

Sir:

We have already reported on the MG values of amino acids in paper-electrophoresis[1,2], which were the constants regardless of voltage, current, time of electrophoresis, dropped point and length of filterpaper. Afterwards, mathematical analysis was applied to the migration distance of amino acids and serum proteins under the influence of evaporation from filterpaper and electroosmosis and it was found that the net-initial velocity of migrant A (B, C, D, E) shown in Fig. 1, $V_{0\,\mathrm{A(B,C,D,E)}}$, could be calculated by the following formula, which was in conformity in a considerable accuracy of 96% (92%~105%), with the net-migation velocity in the filterpaper clamped between glass plates[3].

$$V_{0\,\mathrm{A(B,C,D,E)}}=\frac{(MD'_{\mathrm{A(B,C,D,E)}}-MD'_{\mathrm{dex}})\cdot\log a'/a}{a'/a-1}\quad ..(1)$$

In formula (1), $MD'_{\mathrm{A(E,C,D,E)}}$ shows the apparent migration distance of A (B, C, D, E) from the original point at the time of t_1, MD'_{dex} the apparent migration distance of dextran at the same time, a'/a the decrement ratio of migration velocity of the migrant at the interval of t_1.

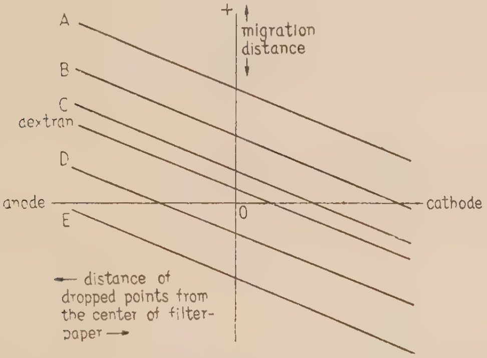
A
B
C
dextran
D
E
+
migration distance
anode
0
cathode
← distance of dropped points from the center of filter-paper →

FIG. 1. The Relationships between the Migration Distance of A (B, C, D, E, Dextran) and Dropped Points[2].

When we take the following ratio as MG of B (C, D),

$$\mathrm{MG}_{\mathrm{B(C,D)}}=\frac{\text{migration distance of B (C, D) from A}}{\text{migration distance of E from A}}$$

then, the MG of B (C, D) is expressed as:

$$\begin{aligned}\mathrm{MG}_{\mathrm{B(C,D)}}&=\frac{MD'_{\mathrm{B(C,D)}}-MD'_{\mathrm{A}}}{MD'_{\mathrm{E}}-MD'_{\mathrm{A}}}=\frac{V_{0\,\mathrm{B(C,D)}}-V_{0\,\mathrm{A}}}{V_{0\,\mathrm{E}}-V_{0\,\mathrm{A}}}\\&=\frac{l/l'\cdot m_{\mathrm{B(C,D)}}-l/l'\cdot m_{\mathrm{A}}}{l/l'\cdot m_{\mathrm{E}}-l/l'\cdot m_{\mathrm{A}}}\\&=\frac{m_{\mathrm{B(C,D)}}-m_{\mathrm{A}}}{m_{\mathrm{E}}-m_{\mathrm{A}}}\quad\cdots\cdots\cdots\cdots(2)\end{aligned}$$

where m_{A}, m_{B}, m_{C}, m_{D} and m_{E} is respectively the mobilities of A, B, C, D and E, l/l' the correction factor of filterpaper for calculation of mobility of migrant[3]. From formula (2) it is evident that the MG value in this definition is equal to the ratio of differences of mobilities, which is constant regardless of voltage, current, time of electrophoresis, dropped point and length of filterpaper. Table I shows the MG values of amino acids and serum proteins, each in accordance with the following definition, which were obtained both by actual measurement under the influence of evaporation from paper surface and by calculation of substituting $l/l'\cdot m$ cm²·volt⁻¹·sec⁻¹ in equation (2), where $l/l'\cdot m$ cm²·volt⁻¹·sec⁻¹ was acquired by a method of clamping filterpaper between glass plates[3].

$$\mathrm{MG}=\frac{\text{migration distance of other serum proteins from }\gamma\text{-globuline}}{\text{migration distance of serum albumine from }\gamma\text{-globuline}}$$

$$\mathrm{MG}=\frac{\text{migration distance of other amino acids from arginine}}{\text{migration distance of aspartic acid from arginine}}$$

We wish to express our sincere gratitude

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[1] K. Sakamoto, K. Tateoka, This Bulletin, **20**, 98 (1956).
[2] K. Sakamoto, K. Tateoka, *J. Agr. Chem. Soc. Jap*, **30**, 463 (1956).
[3] H.G. Kunkel, A. Tiselius, *J. Gen. Physiol*, **35**, 89 (1951).

TABLE I

THE MG VALUES OF AMINO ACIDS OBSERVED IN SEVERAL pH OF PHOSPHATE BUFFERS (A) AND SERUM PROTEINS IN A BARBITAL BUFFER OF pH 8.5 (B)

F: found values C: calculated values from $l/l' \cdot m$

(A)

Amino acid pH		Aspartic acid	Glutamic acid	Neutral amino acid	Histidine	Arginine Lysine
8.3	F	1.00	0.92±0.03	0.34±0.03	0.30±0.02	0
	C	1.00	0.89		0.28	0
7.3	F	1.00	0.87±0.03	0.38±0.03	0.24±0.03	0
	C	1.00	0.85		0.27	0
6.4	F	1.00	0.85±0.04	0.45±0.03	0.18±0.02	0
	C	1.00	0.82		0.17	0
5.5	F	1.00	0.82±0.03	0.50±0.02	0.09±0.03	0
	C	1.00	0.81		0.08	0
4.5	F	1.00	0.78±0.04	0.54±0.03	0.04±0.02	0
	C	1.00	0.79		0.02	0

(B)

		Albumine	α_1-Globuline	α_2-Globuline	β-Globuline	γ-Globuline
MG	F	1.00	0.81±0.03	0.59±0.04	0.35±0.03	0
	C	1.00	0.82	0.56	0.35	0

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Isolation of γ-Phytadiene from the Essential Oil of Tobacco Leaf

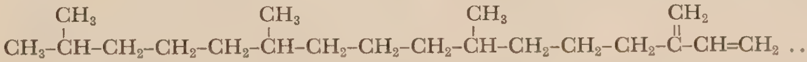
Sir:

We have already reported[1] the presence of an unsaturated aliphatic hydrocarbon resembling myrcene, having a conjugated double-bond in its molecule, contained in the neutral fraction of the essential oil of the aged Japanese Flue-cured tobacco leaf. Further studies were conducted on the structure of this compound which was also separated from the hydrocarbon fraction (H-1)[2] of the aged Japanese Burley tobacco leaf. Molecular weight determination proved that this compound belongs to diterpenes and catalytic hydrogenation indicated that it has two double-bonds in its molecule. Upon decomposition of its ozonide by water, formaldehyde and a monobasic higher fatty acid were obtained. In addition to these results, according to the formation of an aduct with α-naphthoquinone and infrared spectrophotometric investigations of a hydrocarbon separated from the commercially obtained phytol, this compound was estimated as a new hexahydroditerpene (4, 8, 12-trimethyltridecanyl-(1)-2-butadiene-1,3), namely, (7,11,15-trimethyl-3-methylene-hexadecene-1), γ-phytadiene.

Physical and chemical constants of γ-phytadiene:
b.p. 144–146°/3.5 mm: d_{20}^{20} 0.8168: n_D^{20} 1.4628: $[\alpha]_D^{20}+0.03$: M.R. 93.88: M.W. Found by cryoscopic method 269; Calcd. for $C_{20}H_{38}$ 278: *Anal.* Found: C, 86.55; H, 13.25. Calcd. for $C_{20}H_{38}$: C, 86.24; H, 13.75.

Infrared spectrum of γ-phytadiene (Fig. 1) indicated that this compound has two unsaturated radicals which was affirmed to conjugate as $CH_2{=}CR{-}CH{=}CH_2$. Ultraviolet spectrum of γ-phytadiene indicated a maximum absorption at 224 mμ, $\varepsilon = 15475$, and those of myrcene are reported as 224.5 mμ, $\varepsilon = 15350$[3] and 17030[4]. As in the case of infrared spectra, the presence of the conjugated double-bond was also identified by the ultraviolet spectrum.

Ozonization was performed in the chloroform solution and the resulted ozonide was decomposed with water. Formaldehyde (2,4-dinitrophenylhydrazone, m.p. 156°), and a higher aliphatic acid (p-phenylphenacyl ester, m.p. 52.5–53.1°), were obtained from the decomposed product of ozonide. Elementary analysis of this higher aliphatic acid resulted as C_{16} or C_{17}. The infrared spectrum of p-phenylphenacyl ester of this acid is shown



$$CH_3{-}\overset{CH_3}{\overset{|}{C}}H{-}CH_2{-}CH_2{-}CH_2{-}\overset{CH_3}{\overset{|}{C}}H{-}CH_2{-}CH_2{-}CH_2{-}\overset{CH_3}{\overset{|}{C}}H{-}CH_2{-}CH_2{-}CH_2{-}\overset{CH_2}{\overset{\|}{C}}{-}CH{=}CH_2 \quad \ldots\ldots(I)$$

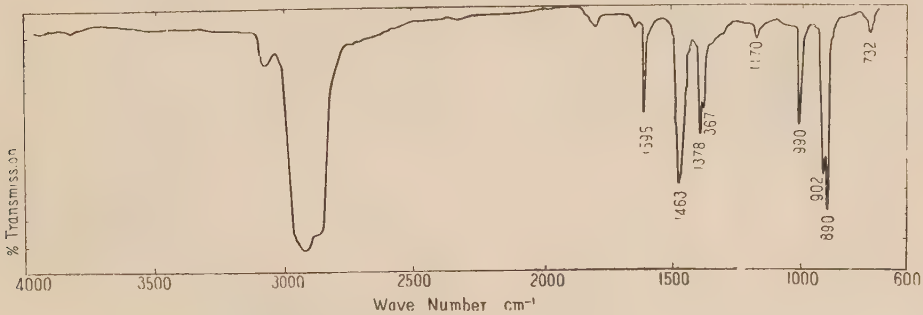


FIG. 1. Infrared Spectrum of γ-Phytadiene.

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[1] I. Ōnishi, H. Tomita and T. Fukuzumi, This Bulletin, **20**, 61 (1956).

[2] I. Ōnishi, M. Nagasawa, H. Tomita and T. Fukuzumi, This Bulletin, (received).

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[4] R.T. O'Connor and L.A. Goldblatt, *Anal. Chem.*, **26**, 1726 (1954).

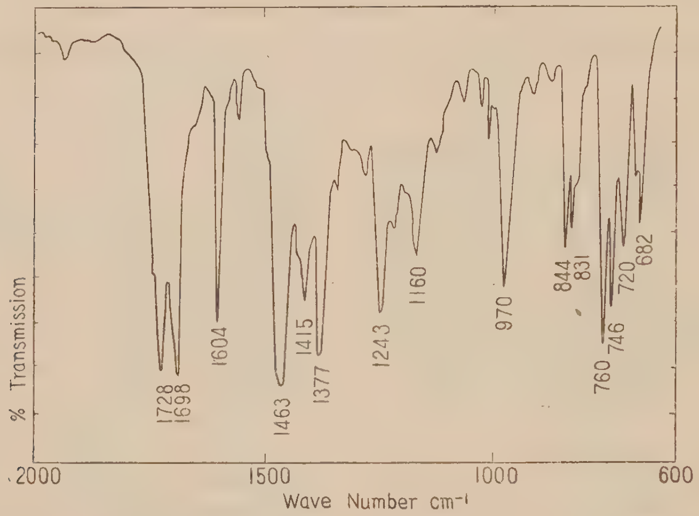

FIG. 2. Infrared Spectrum of *p*-Phenylphenacyl ester of an Acid Obtained by Water Decomposition of γ-Phytadiene-Ozonide (in nujol).

in Fig. 2.

α-Naphthoquinone was reacted with γ-phytadiene and a substance, containing yellow crystals thus obtained, was separated by a liquid chromatography. A white crystal, m.p. 34.5–34.8°, was obtained from the eluate of a yellow band, which was eluted prior to that of the unreacted reagent. *Anal.* Found: C, 82.29; H, 10.06. Calcd. for $C_{30}H_{44}O_2$: C, 82.51; H, 10.16. Infrared spectrum of this aduct is shown in Fig. 3.

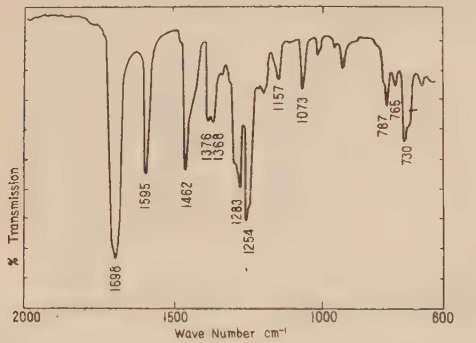

FIG. 3. Infrared Spectrum of an Aduct of γ-Phytadiene with α-Naphthoquinone.

Judging from the infrared spectrum of this compound which indicated the presence of the isopropyl radical at the end of its molecule, and also from other physical properties suggesting that this compound belongs to diterpenes, this compound was supposed to be an isomer of phytadienes5), derived by the dehydrogenation of phytol. The infrared spectrum of a hydrocarbon, separated from the commercially obtained phytol in the purification process, agreed well with that of the above-mentioned newly isolated γ-phytadiene. From these experimental results, the molecular structure of γ-phytadiene (I) was supposed to be reasonable.

The aged Japanese Burley tobacco leaf contains more amount of γ-phytadiene (56.45 mg/kg of leaf) than the aged Japanese Flue-cured leaf (20.06 mg/kg of leaf).

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[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 1, p. 59~60, 1958]

Relationship Between Triseclavine and Isosetoclavine

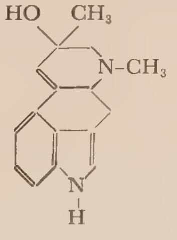
Sir:

We[1], previously, isolated a new alkaloid, triseclavine, from the saprophytic culture of the *Elymus*-type ergot fungus as well as from the culture of the *Agropyrum*-type fungus. After that, we[2] succeeded in isolating a substance, which was identical, at least, in its IR-spectrum with the sample of isosetoclavine generously sent to us by A. Stoll, from the saprophytic culture of another strain belonging to the *Agropyrum*-type ergot fungus.

Isosetoclavine is the alkaloid which was obtained together with setoclavine by A. Stoll and his co-workers[3] in 1955, from the saprophytic culture of the ergot fungus parasitic on African *Pennisetum typhoideum* Rich. Thereupon A. Stoll, considering that isosetoclavine may be identical with our triseclavine, sent the above-mentioned sample to us in the summer of that year and requested us to send a sample of our triseclavine in exchange for the purpose of comparison of the two alkaloids. Naturally, the two alkaloids were compared in both his and our laboratories. Based on comparison of the optical rotation values of the two alkaloids as well as of setoclavine, Stoll has informed us that triseclavine seemed to be a mixture composed of almost equal-parts of setoclavine and isosetoclavine. Our reply to this problem was that triseclavine is a unique alkaloid containing no isosetoclavine. In fact, when triseclavine was developed on Toyo filter paper No. 51 with water-saturated ethyl acetate at room temperature, it gave only a single spot with R_F 0.79–0.82, whereas, the sample of isosetoclavine gave a spot with R_F 0.87–0.89 and though faintly, another spot with R_F 0.79–0.82 corresponding to triseclavine, showing that the sample is an impure substance. From this fact, it was supposed that the so-called setoclavine of A. Stoll et al., must be identical with our triseclavine. This problem may have already been solved by A. Stoll et al., because they possess the two compounds, but they have so far, not informed us about that nor published it anywhere.

At any rate, we[2] already found that oxidation of agroclavine with potassium bichromate in dilute sulfuric acid yields a very small quantity of isosetoclavine besides triseclavine. Proceeding from this finding, it was confirmed for the first time that isosetoclvine is an isomer of triseclavine and that, similar to the latter, it is produced through agroclavine by the oxidative action of fungi. In connection with this, it was found thereafter, that there are two kinds of strains in the *Agropyrum*-type ergot fungus, one of which produces far more triseclavine than isosetoclavine, the other producing far more of latter alkaloid than the former, while the strains belonging to the *Elymus*-type fungus produce triseclavine almost exclusively.

Thus, the problem which still remains unsolved is that concerning the determination of configuration of triseclavine and isosetoclavine, respectively. Recently, in the hope of clarifying this problem, we have examined the chemical and physical properties of these



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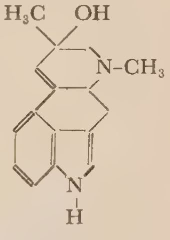

II

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[1] M. Abe, S. Yamatodani, T. Yamano and M. Kusumoto, This Bulletin (communication), **19**, 92 (1955).

[2] M. Abe, S. Yamatodani, T. Yamano and M. Kusumoto, This Bulletin (communication), **20**, 59 (1956); Reported at the Annual Meeting of Agr. Chem. Soc. Japan held at the University of Tokyo (April 1, 1956).

[3] Personal communication.

alkaloids. This examination, especially that of the dissociation constants, led us to believe that the steric constitutions of triseclavine (I) and isosetoclavine (II) must be those shown above. From the result, it seems pertinent to rename isosetoclavine to isotriseclavine.

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Chemical Structure of Gibberellins. Part XI

(Biochemistry of Bakanae Fungus. Part 44)

Sirs:

In our previous reports of this series, we proposed the partial structural formula for gibberellin A_1 shown in Fig. I-1, from the result of ozonolysis and the proton catalysed rearrangement of gibberellin A_1.[1] Moreover, we have pointed out, that the secondary hydroxyl group and the -O- atom of the lactone in ring A of gibberellin A_1 are not vicinal to each other, from the inactivity of the $LiAlH_4$ reduction product of gibberellin A_1 methyl ester towards periodic acid[2].

In this communication, the authors wish to report that the position of the secondary hydroxyl group in ring A of gibberellin A_1 is proved to be in the 2-position of the perhydrated fluorene nucleus as shown in Fig. I-2, from the following experimental results.

The ozonolysis product of gibberellin A_1 methyl ester, $C_{19}H_{24}O_8$, (Fig. II-1a), gives, when reduced by Clemmensen's method with amalgamated Zn and hydrochloric acid, a desoxo-product, $C_{19}H_{26}O_7$, m.p. 250°, (Fig. II-2) (infrared absorption; hydroxyl 3558 cm^{-1}, lactone 1742 cm^{-1}, ester carbonyl 1718 cm^{-1}). The secondary hydroxyl group of this substance is oxidised by CrO_3, with a simultaneous decarboxylation, giving a keto-ester, $C_{18}H_{24}O_5$, m.p. 173–4.5°, (Fig. II-3) (OH-stretching band none, lactone 1766 cm^{-1}, ester carbonyl 1718 cm^{-1} and 6-membered ring ketone 1706 cm^{-1}). When this substance is dehydrogenated with

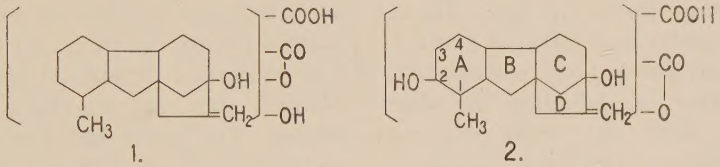

FIG. I.

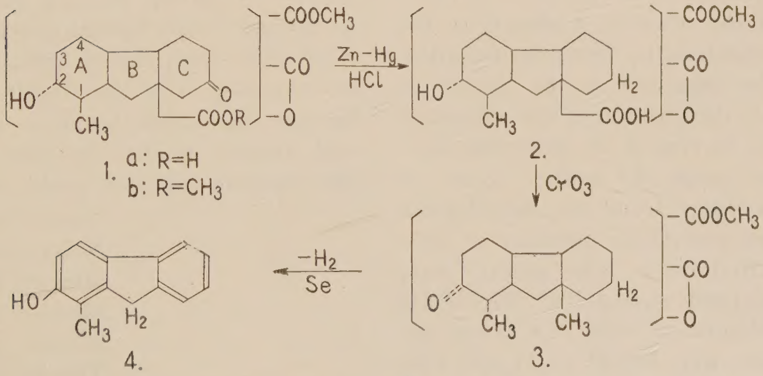

FIG. II.

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[1] Y. Seta, H. Kitamura, N. Takahashi, Y. Sumiki, *Bull. Agr. Chem. Soc. Japan*, **21**, 73 (1957).

[2] N. Takahashi, Y. Seta, H. Kitamura, Y. Sumiki, *Bull. Agr. Chem. Soc. Japan*, **21**, 327 (1957).

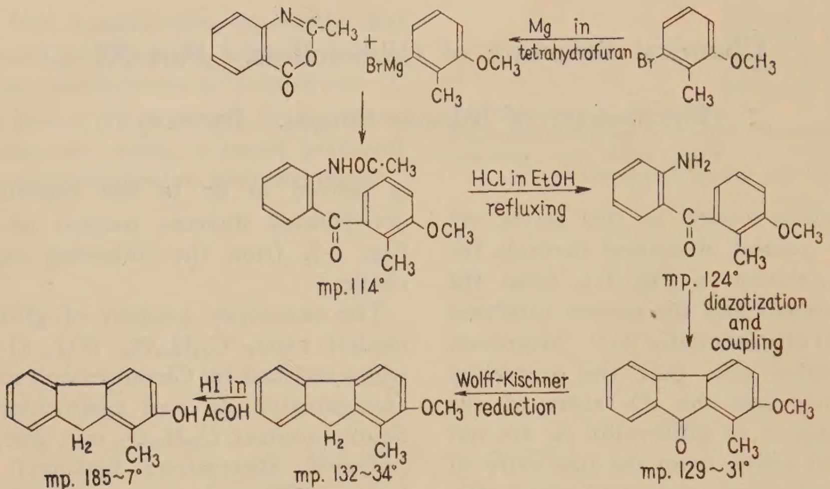

FIG. III.

selenium at 320°, a phenolic substance, $C_{14}H_{12}O$, m.p. 185–7°, is obtained. This has been shown to be identical with 1-methyl-2-hydroxyfluorene, (Fig. II–4) whice was synthesized through the process shown in Fig. III, in mixed m.p., ultraviolet and infrared spectra. As had been already pointed out by Ruzicka[3], Peak and Robinson[4], on selenium dehydrogenation under relatively mild reaction condition, a 6-membered ring ketone remains, as usual, in its ring in the form of phenol. Therefore, in the present case, also, the position of the phenolic hydroxyl in the fluorene thus obtained by selenium dehydrogenation can be regarded as the ketone of Fig. II–3, namely the position of the secondary hydroxyl group in ring A of gibbrellin A_1.

On the other hand, the authors came to the same conclusion from the dehydration reaction of the ozonolysis product of gibberellin A_1 methyl ester. The methyl ester of the ozonolysis product, m.p. 169°, (Fig. II–1b) gives, when dehydrated with $POCl_3$ and pyridine, a product, m.p. 134–6°, $C_{20}H_{24}O_7$, with the elimination of one mole of H_2O. It absorbs one mole of hydrogen on catalytic hydrogenation, and shows infrared absorption at 1758 cm^{-1} (lactone), 1730 cm^{-1} (ester carbonyl), 1704 cm^{-1} (6-membered ring ketone), 1620 cm^{-1} (C=C) and 897 cm^{-1} (=CH_2), indicating the presence of one exocyclic methylene group. Further, on the ozonolysis of this substance, formaldehyde and a neutral product, m.p. 74°, $C_{19}H_{22}O_8 \cdot H_2O$, were obtained. The latter has 5-membered lactonic carbonyl and 6-membered ring ketonic carbonyl absorptions at 1763 cm^{-1} and 1710 cm^{-1}. As the OH group in ring A of gibberellin A_1 had previously been shown to be secondary, it is clear that, this dehydration reaction is accompanied with the simultaneous rearrangement of double bond or methyl radical, and, further studies on the mechanism of this reaction are now under way.

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[4] D.A. Peak, R. Robinson: *J. Chem. Soc.* **1936**, 759.

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